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<p>The research goal is to develop heat shock as a modality for optimizing and controlling gene therapy for treating prostate cancer. Heat activatable tissue permeabilizers will increase dissemination of adenoviral vectors throughout solid tumors. The optimal permeabilizer has been identified and its usable ranges of concentration and activating heat shocks have been narrowed sufficiently to begin testing in human Xenograft tumors. A quantitative relationship between time at temperature and induced expression from a truncated heat shock promoter have been established in exponentially growing and plateau phase prostate cancer cells, which is essential for planning and analyzing parallel experiments in solid tumors. This is a fundamental step for establishing controllable expression of transgenes under control of a heat shock promoter within solid tumors. Mammalian expression constructs for expressing diphtheria toxin A and shiga toxin A have been made and tested, and are currently being used to produce adenoviral expression vectors. Fluorescent chimeras of both proteins have also been made, which may prove useful for monitoring gene expression in tumors and normal tissues. The work presented herein represents considerable progress towards achieving the approved tasks, and establishes a solid foundation for developing a form of controllable gene therapy for treating prostate cancer.</p>				
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*Michael D. Bonnell* 9/27/99  
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## INTRODUCTION

This document is the annual report for Grant DAMD17-98-1-8496 and summarizes the progress made during the first year of funding, viz., September 1, 1998 to August 31, 1999. The research goal is to develop and optimize heat shock as a modality for activating and controlling gene therapy for treating human cancers. Adenovirus vectors will be constructed that contain proteotoxins under control of a modified heat shock promoter. The ultimate goal is to use conformal heating to control the spatial distribution, temporal duration, and the amplitude of proteotoxin expression; thereby maximizing cytotoxicity within tumors while minimizing it in unheated normal tissues. Experiments will be performed in cell culture models and Xenograft tumors to quantitate gene expression in cells under tumor-like physiological conditions, as a function of the temperature and duration of the activating heat shock. Heat shock will also be used to potentiate the activity of chemical permeabilizing agents to increase the penetration and dispersion of adenoviral expression vectors throughout the tumor volume. A selection of different permeabilizing agents will be tested to identify which is most effective and then its optimal concentration for dispersing viruses at 37.0°C or following heat shock will be determined.

## BODY OF REPORT

### **TASK 1: OPTIMIZE DISPERSION OF ADENOVIRUS VECTORS USING TISSUE PERMEABILIZERS**

Spheroids made from a subclone of DU-145 human prostate cancer cells (designated DUT-145) were used to screen three different tissue permeabilizers from Conrex Pharmaceuticals (Phila., PA) to determine which one might prove most effective in promoting virus dispersal in heat shocked tumors. The three tested permeabilizers: formulations 115, 215, and 317 were selected because of their successful application as transdermal drug permeabilizers. Spheroids in three different size ranges were used in these tests, viz., 200  $\mu\text{m}$  to 300  $\mu\text{m}$ , 300  $\mu\text{m}$  to 500  $\mu\text{m}$ , and 500  $\mu\text{m}$  to 750  $\mu\text{m}$ .

The permeabilizers were tested for their ability to increase the intercellular space between cells within the spheroids at both 37.0°C and 41.0°C to determine which provided the biggest differential effect. Spheroids were then infected with adenovirus vectors to determine if treating cells with any of the permeabilizers reduced their ability to be infected by adenovirus vectors.

#### **A. PERMEABILIZER EFFECT ON INTERCELLULAR SPACE AND INTERCELLULAR ATTACHMENT**

##### Permeabilizers #115 and #317

Treatment with permeabilizers #115 or #317 at concentrations of 0.05% to 0.06% for more than 2 h completely dissociated 50% of the spheroids at 37.0°C and 75% at 41.0°C. Spheroids that remained were markedly reduced in size with the actual size range varying considerably from experiment to experiment. More than 65% of the cells were killed following treatments at 37.0°C with 90% killed at 41.0°C. Higher concentrations had more catastrophic effects while decreasing the permeabilizer concentration produced less pronounced effects that were both more reproducible and controllable. Using these permeabilizers at concentrations less than 0.01% had no detectable effect on the spheroids, even following treatments of more than 24 h.

The spheroids remained intact immediately following permeabilizer treatments at 37.0°C or 41.0°C, using concentrations in the range of 0.01% to 0.045%. Spheroids were then infected with adenovirus vectors containing  $\beta$ -galactosidase under control of the CMV promoter and then incubated at 37.0°C for 24 h to 72 h to permit expression of this transgene. During this incubation time the outer cell layers of many spheroids treated with permeabilizer concentrations of 0.025% to 0.045% became detached from the spheroids, with the magnitude of detachment being proportional to increasing permeabilizer concentration. The majority of these detached cells exhibited  $\beta$ -galactosidase expression indicating that the permeabilizers promoted deeper penetration of the adenovirus into the spheroids.

The remaining spheroids fell apart completely or partially during processing for embedding and sectioning. This was reduced somewhat by fixing the spheroids longer with glutaraldehyde, but this obviated the ability to stain for  $\beta$ -galactosidase activity or locate  $\beta$ -galactosidase by immunohistochemistry. Thus, the dissociation of cells from the spheroids prevented us from quantitating the depth of adenovirus penetration into the spheroids, as planned originally. Cells within spheroids made from MCF-7 human breast carcinoma cells were markedly more resistant to dissociation from the spheroid following permeabilizer treat-

ment. Presumably, the differences observed between DUT-145 and MCF-7 cells occurred because the two cell types exhibit different levels of cell-cell binding within the spheroid

#### Permeabilizer #215

Spheroids treated with permeabilizer #215 exhibited a more pronounced increase in the effects obtained at 41.0°C relative to those at 37.0°C than was observed for the other two permeabilizers. The permeabilizer-induced dissociation of cells from the spheroids was less pronounced at a given permeabilizer concentration. Unfortunately, the spheroids still dissociated while they were incubated at 37.0°C to express  $\beta$ -galactosidase; sufficiently enough that depth of virus penetration could not be quantitated in sectioned material.

Experiments were continued only long enough to ascertain the concentration range of permeabilizer #215 that increased the intercellular space, and/or caused cell detachment from the spheroids, without killing cells. The usable concentration range was determined at 37.0°C and as a function of time at 41.0°C. Table 1 summarizes these data.

**TABLE 1: OPTIMAL PERMEABILIZER #215 CONCENTRATION AS A FUNCTION OF TIME AT TEMPERATURE**

TEMPERATURE	TIME AT TEMPERATURE			
	1-2 h	6 h	12 h	24 h
37.0°C	0.125%	0.08%	0.06%	0.04%
41.0°C	0.05- 0.07%	0.04%	0.025%	0.01%

#### Summary

These experiments did not provide quantitative data concerning the ability of the permeabilizers to increase the penetration depth of adenovirus into spheroids. However, they did provide the means to identify the optimal permeabilizer and then narrow the range of parameters (permeabilizer concentration and time at temperature) that will be tested within Xenograft tumors expressed in nude mice. In this respect the experimental results were very important, as they will significantly reduce the number of nude mice that will be required for the in vivo permeabilizer experiments.

#### **B. EFFECT OF PERMEABILIZER #215 IN RAT TUMORS IN VIVO**

Dr. Elwood Armour, a colleague at William Beaumont Hospital, had six spare rats each having two to four 9L cell tumors (approx. 1 cm diameter each). These animals were intended as controls for an experiment that failed, and were scheduled to be euthenized. We petitioned Dr. Armour to use these animals for testing the effects of permeabilizer #215 on solid tumors. Dr. Armour agreed, and the William Beaumont Hospital Animal Care Committee gave permission for the experiments since testing the permeabilizer fell under the approved protocol that Dr. Armour had for these animals.

Permeabilizer #215 was diluted into cell culture medium (no serum) and injected into the tumors using long 28 g needles. The needles were pushed into the tumors and the permeabilizer solution was injected into the needle tract as the needle was withdrawn. At least four injections were made in each tumor. Sham injections consisted of introducing culture medium

but no permeabilizer. Tumors were either heated to 41.0°C using a broadly focused ultrasound transducer (thermocouple feedback control) or sham heated (no power to transducer). After euthanizing the animals the tumors were removed, cut in half, frozen, and then cryosectioned.

Figure 1a shows a cryosection taken transverse to a needle tract in a control tumor, i.e., culture medium injection and no heat. The needle tract appears as a clear void and the adjacent tissue maintained a morphology characteristic of that found in the control, undisturbed, tumors. The section in Figure 1b was taken parallel to a needle tract into which a 0.1% solution of permeabilizer #215 was injected. The tumor was harvested 2 h after injecting the permeabilizer and the tissue adjacent to the needle tract appears similar to that seen in the control section of Fig. 1a. Figure 1c shows a section taken transverse to a needle tract into which a 0.1% solution of permeabilizer #215 was injected. The tumor was then heated for 2 h at 41.0°C and harvested immediately afterwards for cryosectioning. The needle tract appears as an enlarged void and the adjacent tissue appears abnormal with many eddies and channels through it. It is postulated that these eddies and channels will provide the means for adenovirus vectors to penetrate deeper throughout heat shocked tumors treated with the permeabilizer. It is expected that we will end up using permeabilizer concentrations lower than the 0.10% used for this experiment. However, the results with this higher permeabilizer concentration clearly illustrate the ability of heat to potentiate the permeabilizer's effects on tissue.

Although the needle tract in Fig. 1c appears as a large void, the material was actually a clear, solid, gelatinous mass. It was as if the cells dissolved into the permeabilizer, with perhaps released DNA giving the material a very viscous, gelatinous consistency.

## SUMMARY

Permeabilizer #215 had very little effect on tumor cells and morphology following a 2 h treatment at normal body temperature. However, a 41.0°C markedly increased its effect on tissues, literally causing a 0.10% solution to permeabilize and apparently solubilize tumor cells into a solid gelatinous mass near the needle tract through which it was delivered. The tissue damage decreased progressively as the radial distance from the needle tract increased, ostensibly because the concentration of the permeabilizer decreased proportionately. The less damaged tissue showed channels and increased intercellular spaces that could facilitate adenovirus penetration throughout tumors, as postulated. Clearly, permeabilizer concentrations of less than 0.10% will have to be used if the latter is the sole objective. However, the data in Fig. 1 suggest that the permeabilizer might also be used as a potent heat sensitizer that can debulk a tumor by solubilizing tumor cells when they are heated. The fact that the destroyed cells congeal into a solid, gelatinous mass may be advantageous for inhibiting metastases of tumor cells from the debulked tumor.

**TASK : ESTABLISH THE QUANTITATIVE RELATIONSHIP BETWEEN THE MAGNITUDE OF THE INDUCING HEAT SHOCK (TIME AT TEMPERATURE) AND THE RESULTANT LEVEL OF TRANSGENE EXPRESSION FROM THE HEAT SHOCK PROMOTER IN XENOGRAFT HUMAN PROSTATE TUMORS.**

### **A. PRELIMINARY TESTS IN A RAT, WINDOW-CHAMBER TUMOR MODEL**

During the initial months of this study Dr. Mark Dewhirst, from the Duke University Department of Radiation Oncology, offered us the opportunity to test out some of our adenovirus vectors in his rat, window-chamber, tumor model. We recognized this as an excellent opportunity to gain data that would help study once we began experimenting on Xenograft tumors



in nude mice. During the first year of this study we have utilized this window chamber model on several occasions.

The model system is illustrated in Figs. 2a and 2b, with the former showing a schematic of the window chamber and how the virus vectors are introduced, and the latter showing the window chamber attached to a female Fischer 344 rat. The tumor used in these experiments grown from cultures of rat mammary adenocarcinoma cells (MACs). These tumors were infected with adenoviruses containing enhanced green fluorescent protein (EGFP) under control of the CMV promoter of the truncated human heat shock promoter. The adenovirus vector containing EGFP under control of the heat shock promoter was designated Adv-AHSP-EGFP.

A considerable amount of data was obtained, and will be used to produce a manuscript that will be sent to Cancer Research. A sample of the data that we obtained is presented in Fig. 4.

There is one aspect of the data in Fig. 4 that stood out: The EGFP fluorescence was nonuniform throughout the tumor. We thought this unusual since we anticipated that adenovirus dispersal throughout such a thin tumor would be relatively uniform. Phase contrast images of these tumor regions showed that the areas that initially exhibited lower EGFP fluorescence had very high concentrations of cells. It was postulated that cells in these regions might be more plateau-like than other regions of the tumor. Consequently, we deemed it necessary to quantitate transgene expression from the heat shock promoter within human prostate cancer cells in the different growth phases that would be encountered in a tumor. The next section presents the results of this quantitative study using either exponential or plateau phase cultures of DU-145 cells infected with adenovirus vectors to deliver the transgenes.

## **B. QUANTITATIVE STUDY OF TRANSGENE EXPRESSION IN ADENOVIRUS-INFECTED CULTURES OF EXPONENTIALLY GROWING OR PLATEAU PHASE DU-145 CELLS.**

The data from the window chamber experiments indicated that we needed to determine the quantitative relationship between the magnitude of the inducing heat shock and the magnitude and temporal duration of transgene expression by the heat shock promoter. These experiments were performed in a more simple culture model, viz., monolayer cultures of DU-145 cells growing either exponentially or in plateau phase. We felt strongly that the resultant data would prove invaluable in planning experiments in the Xenograft tumors and in interpreting the resultant data.

### **Expression from the Heat Shock Promoter in Exponentially Growing Cells**

As long as the MOI was kept under 60, Du-145 cells infected with adenovirus vectors that introduced recombinant DNA under control of the truncated heat shock promoter exhibited no detectable expression of that recombinant DNA unless they were heat shocked. This is illustrated by both photo micrographs (Figs. 5 and 6) and Western Blots (Fig. 7 and 8). These same figures also illustrate that the level of expressed enhanced green fluorescent protein (EGFP) was proportional to the MOI and both the temperature and duration of the heat shock. Again, no expression was observed in unheated, infected DU-145 cells, or in unheated, infected cultures of three other tested cell lines (A549 human lung carcinoma, HeLa, and WIDR human colon carcinoma) (data not shown).

Expression was barely detectable following heat shocks at 40.5°C and maximal expression was attained using heat shocks at 41.0°C or 42.0°C. Exposures at 43.0°C (Figs. 7 and 8)

and higher temperatures (data not shown) resulted in progressively lower levels of EGFP expression during the first 24 h following heat shock. Ostensibly, this occurred because the higher temperature heat shocks markedly reduced transcription and translation and produced significant cell killing.

Temporal profiles of heat-induced EGFP expression in adenovirus-infected cells are presented quantitatively in Figures 9 a-d. Cells were heated at 41.0°C or 42.0°C 24 h following virus infection since preliminary experiments demonstrated that this produced the greatest induced EGFP expression. These figures demonstrate that the duration of the heat shock had a significant effect on the magnitude of EGFP expression, particularly at the peak levels. However, the increase in peak levels of cellular EGFP content was not linear with increasing heating time. Once heating times at 42.0°C exceeded 3 h, longer heating times resulted in progressively lower EGFP expression, ostensibly because the more severe heat shock inhibited transcription and/or translation, or even killed some of the cells. Interestingly, the duration over which induced EGFP could be detected remained relatively refractory to heating time or the use of 41.0°C or 42.0°C for the heat shock.

The spectrofluorimeter determination of EGFP proved usable over a wider range of cellular EGFP levels than using quantitative Western Blots (data not shown), possibly because the linearity between EGFP levels and fluorescence was more expansive than that for film density for exposed protein bands on chemilumigraphs. Because of this, and its greater ease of measurement (especially for numerous samples) and better reproducibility, the spectrofluorimeter method of quantitating cellular EGFP was utilized throughout the study. Spot checks of the spectrofluorimetric data by quantitative Western Blots showed good agreement between the two methods (data not shown).

Temporal profiles of EGFP expression from cells infected with viral vectors containing EGFP under control of the CMV promoter are presented in Fig. 10a to compare expression from the two different promoters. A very obvious feature of this figure is the marked nonlinearity between MOI and peak cellular EGFP levels. The MOI 30 data was included in this figure to emphasize this characteristic. This nonlinearity was not due to nonlinear viral infection rates. Figure 10b clearly shows that introduction of viral DNA into cells by infection with adenoviruses containing H<sup>3</sup>-thymidine labeled DNA was linear with MOI. Consequently, the nonlinearity in cellular EGFP levels had to occur at the transcriptional or translational level. Recently acquired Northern blot data shows that the nonlinearity is at the transcriptional level (data not shown).

The data showed that EGFP expression following an MOI 10 infection with virus containing EGFP under control of the CMV promoter was very low and remained essentially constant between 24 h and 100 h following infection. The heat shock promoter outperformed the CMV promoter at this MOI for all the heat shocks used. Over a 24 h period the CMV and heat shock promoters resulted in comparable levels of EGFP per cell, especially between 24 h and 48 h post infection. The latter time frame is most relevant for comparison since cells were heat shocked 24 h following infection and peak EGFP levels were observed about 24 h following the heat shocks. In actuality, the heat shock promoter out produced the CMV promoter over a 24 h period at MOI 20. This is because prior results have demonstrated that the heat shock promoter was not on continuously, but rather decreased its activity rapidly following peak activity at 3 to 6 h following heat shock (data not shown).

Figure 11a illustrates that the heat shock promoter can be induced to express EGFP at any time within the same temporal span in which EGFP was expressed constitutively by the

CMV promoter. However, the peak inducible EGFP expression decreased with heat inductions that were delivered later than 24 h following viral infection. When successive heat shocks of 2 h at 41.0°C were delivered every 12 h following virus infection the level of EGFP expressed from the heat shock promoter was both increased and protracted (Fig. 11b). The resultant cellular level of EGFP was approximately twice that resulting from one 2 h 41.0°C heat shock and this remained essentially constant for more than 96 h following the initial heat shock. In cells receiving a single heat shock (Fig. 2a-d) cellular EGFP decayed to about 30% of the peak level within 72 h following the heat shock. Consequently, if one heat shock fails to produce sufficient gene product, or to maintain the gene product at a desired level for a specified time period, Fig. 11b suggests that utilizing subsequent heat shocks could provide the desired level and temporal duration of expression from the heat shock promoter over a protracted time period.

#### Expression from the Heat Shock Promoter in Plateau Phase Cells

Human tumors consist of cells in plateau phase growth as well as the exponentially growing cells utilized for the previously presented data. Likewise, most normal tissues do not grow exponentially. Consequently, it was deemed necessary to repeat some experiments using cells that were maintained as plateau phase cultures during viral infection and EGFP expression. Figure 12 clearly shows that EGFP expression under control of the CMV promoter in plateau phase cultures increased more slowly and never achieved less than half of the peak expression level observed in exponentially growing cells. However, cellular EGFP remained at the peak levels longer, showing little decrease even more than 200 h following virus infection.

The time period over which EGFP expression from the heat shock promoter could be induced was markedly increased in plateau phase cultures (Fig. 13). Peak EGFP expression was similar to that observed in exponential cultures, and showed a lower proportional decrease than that for EGFP expressed from the CMV promoter. However, peak expression was achieved when cells were heated 48 h following infection rather than at 12-24 h following infection, as in exponentially growing cells. Interestingly, the decay from the peak EGFP level was very similar to that observed in exponentially growing cells Fig. 14. Catabolic degradation of the EGFP (and other cellular proteins) to provide amino acids and other materials for the nutrient-deprived, plateau phase cells may have caused the EGFP decrease since protein dilution by cell division would have been extremely slow.

#### Summary

Induced EGFP expression from the heat shock promoter was measured in adenovirus-infected, exponentially growing and plateau phase cultures of DU-145 cells. Clearly, plateau phase had a significant effect on EGFP expression from both the heat shock and CMV promoters. The kinetics of EGFP from expression from both promoters were reduced in plateau phase cultures relative to those in exponential cultures. However, the peak level of expression attained in plateau cultures was reduced to a lesser degree than EGFP expressed from the CMV-promoter.

It is anticipated that the data obtained from the exponential and plateau phase monolayer cultures help significantly in planning similar experiments in Xenograft tumors and be an aid in interpreting data from the tumor experiments.

### **C. HEAT SHOCK-INDUCED UPREGULATION THE CMV PROMOTER ACTIVITY**

During the quantitative experiments described above, we attempted to use cells infected with adenovirus vectors containing EGFP under control of the CMV promoter as a negative control for heat-upregulation of the heat shock promoter. What we observed was a marked increase in CMV promoter activity that was proportional to the heat shock used. The heat-induced increase was nearly thirty-fold for cells infected at an MOI-20 with adenovirus to express EGFP by the CMV promoter. We and others have seen a mild heat-induced increase in CMV activity, but never one so large. One possible explanation may be that the effect is greatly enhanced in tumor cells versus normal cells. For example, the heat-induced upregulation of the CMV promoter was only three-fold for an identically treated normal human prostate cell line (PZ-HPV-7).

This is an interesting phenomenon that will be investigated further, as it does fall under our statement of work to develop heat-activated forms of gene therapy. It may provide the means to have a basal expression level of a therapeutic transgenes that can be upregulated in selected tissue regions using conformally delivered heat shocks. The future work will include ascertaining whether or not the heat potentiation of the CMV promoter is universally greater in cancer cells than in normal cells.

### **D. UPREGULATION OF CMV AND SV-40 PROMOTER ACTIVITY BY UNIDENTIFIED ADENOVIRUS GENES**

In several experiments, adenoviruses made from the PFG-140 plasmid were used as negative controls for adenoviruses expressing transgenes. This virus was selected because it was easily made and had no transgene material. It does, however, contain adenovirus genes that are not present in adenoviruses that contain recombinant transgenes. The reason for this is that these "nonessential" adenoviral genes had to be removed to make room for the transgenes.

Figure 16 shows that the PF-140 virus markedly enhances expression of transgenes under control of the CMV promoter. Similar data were obtained for transgenes under control of the SV-40 promoter, but not the human beta-globulin promoter. This suggests the postulate that the PFG-140 enhancement effect only occurs with transgenes under control of viral promoters.

There is significant therapeutic potential for this enhancement effect. One application is to use PFG-140 virus as an adjuvant for expressing transgenes on other adenoviruses. If large quantities of this one virus are produced, smaller amounts of other, transgene viruses will need to be produced. Desired levels of transgene expression can be achieved with less virus by using this "Universal" adjuvant virus.

### **TASK 3: CONSTRUCT AND TEST ADENOVIRUS VECTORS TO EXPRESS THERAPEUTIC GENES UNDER CONTROL OF THE TRUNCATED HEAT SHOCK PROMOTER**

#### **A. ADENOVIRUS EXPRESSING DIPHTHERIA TOXIN A**

Three different mammalian expression constructs for expressing diphtheria toxin A (DTXA) were produced and placed into plasmids that can ultimately be used to make adenovirus vectors. One construct expressed DTXA by the CMV promoter (CMV-DTXA) and another expressed DTXA under control of the truncated heat shock promoter (AHSP-DTXA).

The third construct was a chimeric protein of EGFP fused to the N-terminus of DTXA and expressed by the CMV promoter (CMV-EGFP-DTXA).

Prior to making adenovirus vectors, each of these constructs was tested by electroporating the respective plasmids into A549 and DU-145 cells. Electroporation is used routinely in our laboratory and consistently results in the transfection of recombinant DNA into 50 to 70% of the cells in each sample.

Figure 17 shows the results of an experiment with A549 human tumor cells transfected with the CMV-EGFP-DTXA construct. Cells were plated on 500 $\mu$ M photoetched cover slips immediately after electroporation with 30  $\mu$ g of DNA and allowed to settle for three hours prior to rinsing with fresh medium to remove debris. Serial image analysis with phase contrast and epifluorescence was performed over the next for 36 hours to determine any resultant toxicity. Figure 17A is the phase contrast image at 8 hours post transfection and Figure 17B is the corresponding exact register epifluorescence image to detect EGFP. At the three hour time point the corresponding phase contrast image showed 196 cells, which all exhibited normal morphology. Eight hours after transfection many of the cells were rounded, blebbing and with picnotic nuclei. One hundred and twenty eight cells (65%) were detected by epifluorescence and the fluorescence corresponded precisely with the position of dying cells. All fluorescing cells died within the 36-hour period of serial time lapse image analysis while most, but not all, non-fluorescing cells proliferated normally. This observation is considered proof of the expression and toxic function of the CMV-EGFP-DTXA fusion protein construct. The white arrows in Figure 20 point to corresponding cells in both images and are positioned to assist readers in registering the images. Transfection with a CMV-EGFP construct plasmid did not effect normal proliferation or morphology over a 36 hour period (data not shown).

Figure 18 shows cell killing in heat shocked cells transfected with a plasmid containing DTXA under control of the truncated heat shock promoter (Fig. 18 e and 18 f). If no heat is administered to activate the heat shock promoter cells transfected with the AHSP-DTXA plasmid do not die and proliferate normally. The toxic effects of the CMV-DTXA construct (data not shown) were identical to that of the CMV-EGFP-DTXA construct.

We are currently using these DTXA constructs to produce adenovirus vectors that will express DTXA in infected cells either constitutively by the CMV promoter or following an inducing heat shock by the heat shock promoter.

## **SUMMARY**

We have made good progress with the DTXA constructs and anticipate having usable adenovirus vectors and testing them by the end of the year.

### **B. ADENOVIRUS EXPRESSING SHIGA TOXIN A**

Although we made shiga toxin A (STXA) constructs in a similar manner as those containing DTXA, we did not have the same success that we had with the DTXA constructs. Following transfection we found little or no toxicity exhibited by the transfected cells. An analysis of the STXA DNA sequence revealed a potential problem that was not present in the DTXA sequence. The former had three large stretches of codons that are found very infrequently in mammalian cells. Past experience has indicated that this could result in little or no expression of the STXA coding sequence in mammalian cells.

To circumvent this obstacle, we had the STXA cDNA re-manufactured by OPERON Technologies, Inc. (Alameda, CA) to replace the infrequent codons with ones found more frequently in mammalian cells. Dr. Peter Corry provided the \$5,346 required for this process.

OPERON performed a careful secondary structure analyses to ensure that the proposed "mammalianized" codon changes did not produce aberrant secondary DNA structures that would interfere with transcription of the STXA cDNA. The resultant product had most of the infrequent codons re-coded and had no detectable, abnormal, secondary DNA structure

The mammalianized STXA cDNA has been placed into the same plasmids that were used for the DTXA. Initial tests indicate that the mammalianized STXA produces a toxic product in A549 and DU-145 cells (data not shown).

## KEY RESEARCH ACCOMPLISHMENTS: YEAR ONE

- Identified permeabilizer #215 as the tissue permeabilizer that is most likely to function best within Xenograft Tumors made from human prostate cancer cells.
- Determined the optimal concentration range for using permeabilizer #215 with spheroids or in tissues at 37.0°C or 41.0°C to increase the intercellular space.
- Observed that the tissue permeabilizers, at higher concentrations, solubilized cells in solid tumors heated to 41.0°C with no detectable effect at normal body temperature. This suggests the possibility that the permeabilizers might serve as heat sensitizing agents for killing tumor cells with heat shock.
- Demonstrated that the heat-activated reporter gene Adenovirus vectors work in a window-chamber, tumor model.
- Established a quantitative relationship between the magnitude of the inducing heat shock (time at temperature) and the magnitude and temporal duration of the induced transgene expression by the truncated heat shock promoter in human prostate cancer cells. This was accomplished for cells growing exponentially and in plateau phase.
- Discovered that heat shock enhancement of transgene expression by the CMV promoter may provide a controlled means of upregulating basal CMV expression to significantly increase transgene products in selected tissue sites. Very preliminary data suggest that this CMV promoter upregulation may occur preferentially in tumor cells.
- Discovered that co-infecting cells with the PFG-140 virus markedly increases transgene expression by both the CMV and SV-40 promoters. No such increase was observed for the two human promoters that were also tested, viz., the  $\beta$ -globulin and heat shock promoters.
- Made functional mammalian expression constructs for diphtheria toxin A (DTXA) under control of the truncated heat shock promoter and CMV promoter. The former constructs were toxic only in heated cells. One of these constructs was a fluorescent chimera of EGFP and DTXA that may prove useful in monitoring transgene expression within solid tumors.
- Successfully reconstructed the shiga toxin A (STXA) cDNA to circumvent the inability to express this protein in mammalian cells due, putatively, to large tracts of infrequent mammalian codons within the cDNA.

## **REPORTABLE OUTCOMES**

### **Manuscripts**

The data presented herein, and related data, are currently being used in the writing of three manuscripts. The first manuscript will report the quantitative analyses of transgene expression in adenovirus-infected prostate cancer cells, for transgenes under control of the heat shock or CMV promoters. This manuscript is being targeted for Cancer Research. A second manuscript will report the work with the window-chamber tumor model that we did in collaboration with Dr. Mark Dewhirst. Again, this is being targeted for Cancer Research. Finally, a third manuscript is being prepared to report the heat upregulation of the CMV promoter and the ability of the PFG-140 virus to increase expression from both the CMV and SV-40 promoters.

### **PATENTS**

We are currently working with Conrex Pharmaceuticals to develop a patent for using the tissue permeabilizers to facilitate adenovirus vector delivery to larger tissue volumes.



## CONCLUSIONS

### TASK 1

Initial experiments have identified Conrex permeabilizer #215 as the permeabilizing agent with the greatest potential for making solid tumors more permeable to adenovirus vectors when tumors are heated to 41.0°C. It has the least observable effects at 37.0°C and exhibits the biggest differential response following heat shock.

Permeabilizer #215 will now be tested in human prostate cancer Xenograft tumors in nude mice. If this compound performs as expected it would help surmount a major obstacle to successful gene therapy, viz., delivery of viral vectors to a sufficiently large tissue volume to produce a clinically relevant effect on diseased tissues. Success with permeabilizer #215 would have a significant impact on all aspects of gene therapy, not just those applications for treating cancer, since many gene therapy applications share this same impediment.

The spheroid experiment results were not as clean as desired. This was due to the unforeseen complication of the DUT-145 spheroids dissociating more completely than expected following protracted treatment with the permeabilizers and during subsequent histological preparations. Other spheroids, e.g., those made from MCF-7 cells, have proven more resistant to this dissociation. However, using these cells is not germane to this study into developing prostate cancer treatments. Other funding sources will be sought to pursue additional spheroid work with MCF-7 cells. Meanwhile, preliminary experiments indicate that the surrounding tissue will keep cells from the DU-145 tumors confined to the tumor volume following permeabilizer treatments.

### TASK 2:

Prior to the work presented herein, there was no comprehensive study quantitating the magnitude of recombinant DNA expression from the truncated heat shock promoter as a function of heat dose (time at temperature). These data will be used to determine the heat shock exposures required to induce desired levels of transgene expression from the heat shock promoter in solid tissues. Initial experiments will ascertain how accurately the *in vitro* data predict *in vivo* results. Of particular interest will be if the *in vitro* differences between induced transgene expression in exponentially growing cells and plateau phase cells translates into regional differences in expression due to variations in tumor physiology and cell cycle distribution of a tumor's cell population.

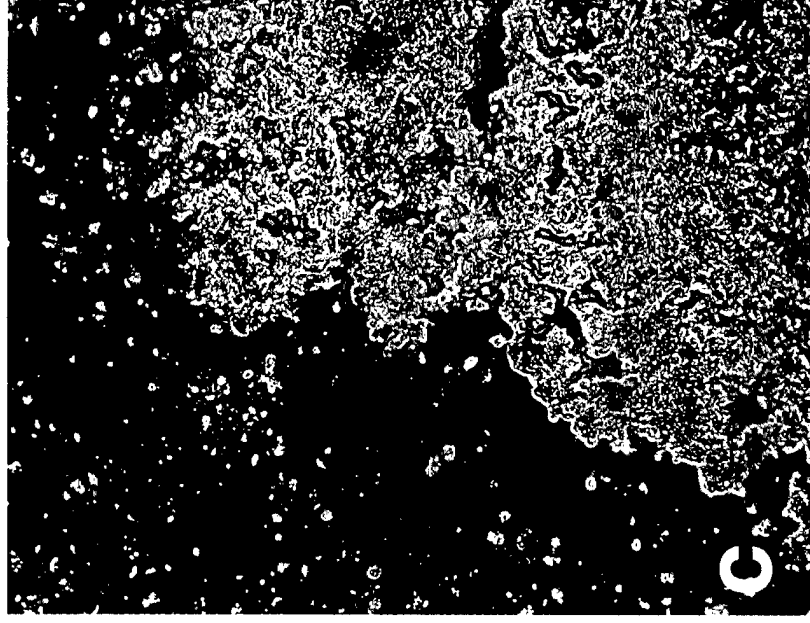
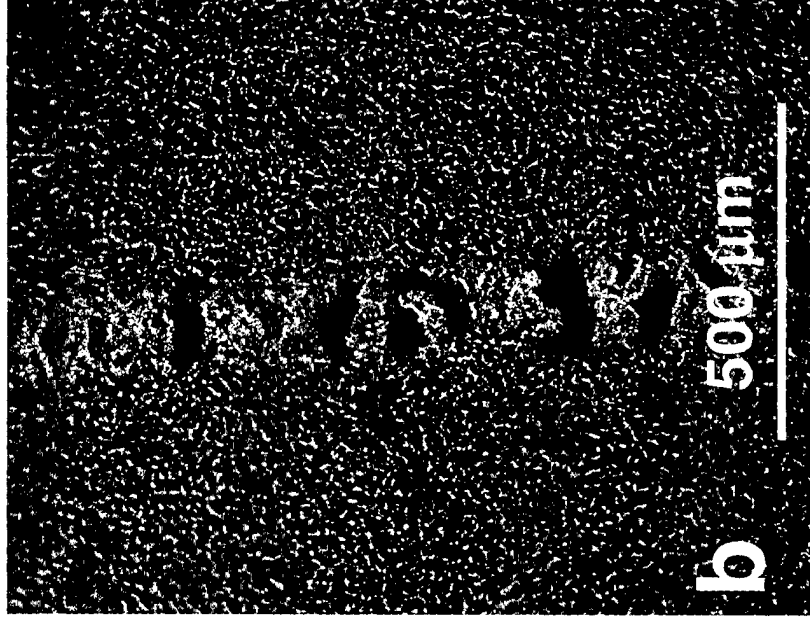
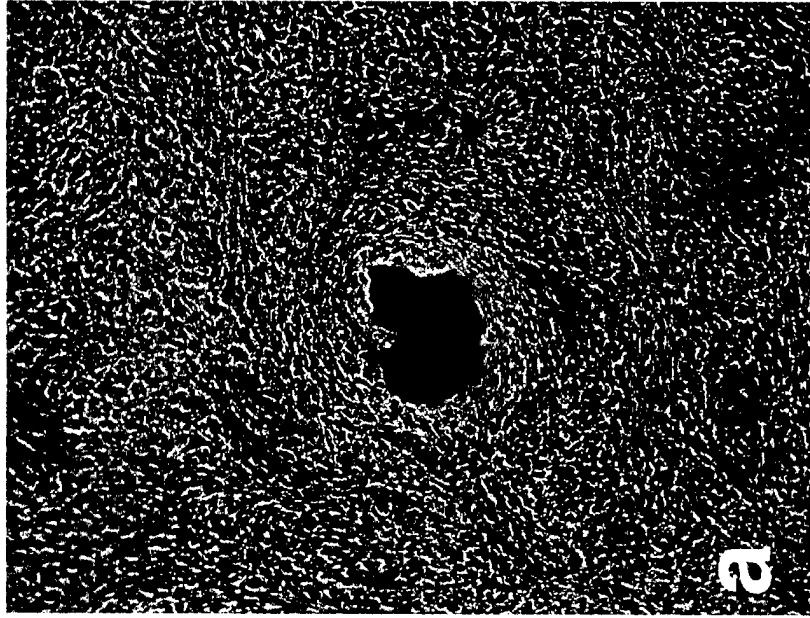
### TASK 3

Obtaining adenovirus vectors expressing proteotoxins under control of the truncated heat shock promoter is a critical goal of this study. Successfully constructing expression cassettes for diphtheria toxin A (DTXA) and shiga toxin A (STXA) is a major step in accomplishing the final goal. This is especially true in light of the unanticipated difficulties encountered in making constructs that expressed STXA in mammalian cells. A similar sequence analysis will be performed for all other proteotoxin genes or cDNA prior to using them in mammalian expression constructs to determine if infrequent codon blocks will present problems with expression.

Should we encounter difficulty in making the desired adenovirus vectors, we will investigate using other cytotoxic compounds in the adenovirus vectors, e.g., cytokines, interleukins, etc.

## **SIGNIFICANCE**

Gene therapy holds great promise as a powerful clinical treatment for human diseases. Like many new and developing therapies, there are many obstacles that must be surmounted before gene therapy becomes an effective and commonplace treatment. Among these are delivery of sufficient recombinant DNA into the diseased tissue and controlling gene expression once it has begun. Our research into developing the concept of heat-activated gene therapy has the potential to help solve both of these impediments to successful gene therapy. Heat shock can be used to potentiate the activity of tissue permeabilizers that can facilitate dissemination of adenovirus expression vectors throughout the heated tissue volume. Once the therapeutic genes are in place, heat can then be used to define the spatial volume within which the heat shock promoter will be activated to express the therapeutic genes. Furthermore, the magnitude of the heat shock can be varied both spatially and temporally to provide finer and more comprehensive control over the spatial and temporal expression of therapeutic genes. Ostensibly, more treatment can be delivered to those tissues needing it most, while potentially adverse side effects in surrounding normal tissues can be minimized using conformal heating techniques that will not activate therapeutic genes in normal tissues.



**FIGURE 1: EFFECT OF ENHANCER #215 IN RAT 9L SOLID TUMORS AT BODY TEMPERATURE OR 41.0°C**

- a) Control Tissue at normal body temperature. Section was taken transverse to a needle tract into which cell culture medium was injected. This represents normal tumor morphology. Tumor was harvested 2 h after needle injection.
- b) Tissue injected with a 0.10% solution of permeabilizer #215 and maintained at normal body temperature. Section was taken parallel to the needle tract to show morphology of tissue adjacent to needle tract, which appears normal. Tumor was harvested 2 h after injecting permeabilizer.
- c) Tissue heated for 2 h at 41.0°C immediately after injecting a 0.10% solution of permeabilizer #215. This section was taken transverse to the needle tract. Note the severely damaged tissue in the vastly enlarged needle tract and the abnormal morphology of the tissue adjacent to the tract.

Figure 2. Experimental setup of the rat window chamber model system. a) Diagrammatic representation of the dorsal flap chamber shown pictorially in panel b, attached to a Fischer 344 rat. Adenoviral vectors were injected from the side of the viewport.

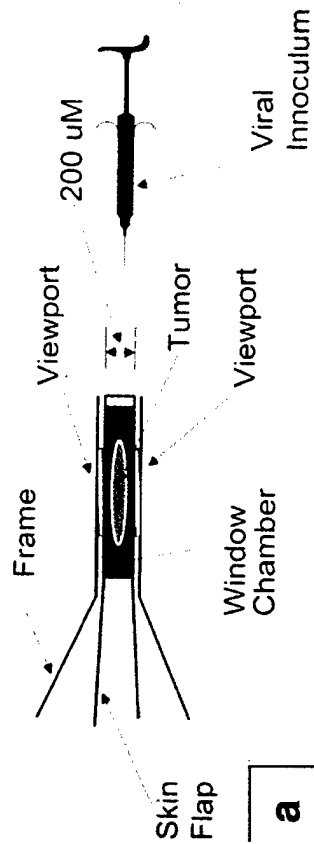
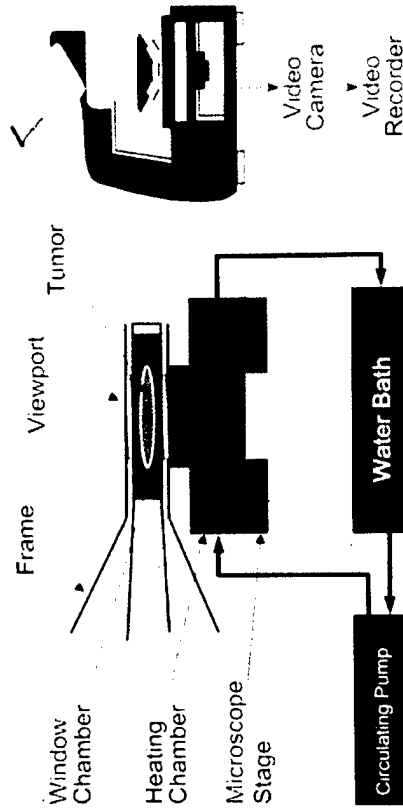
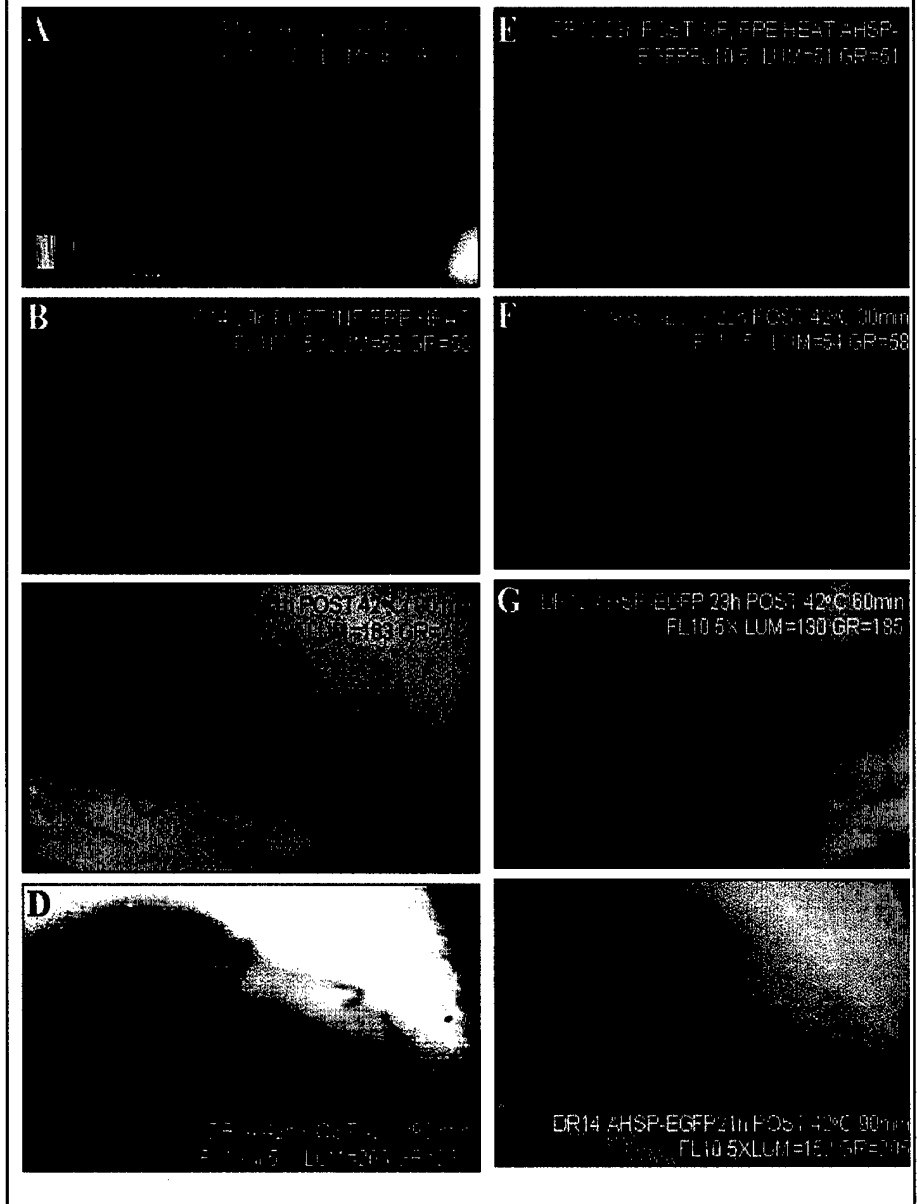
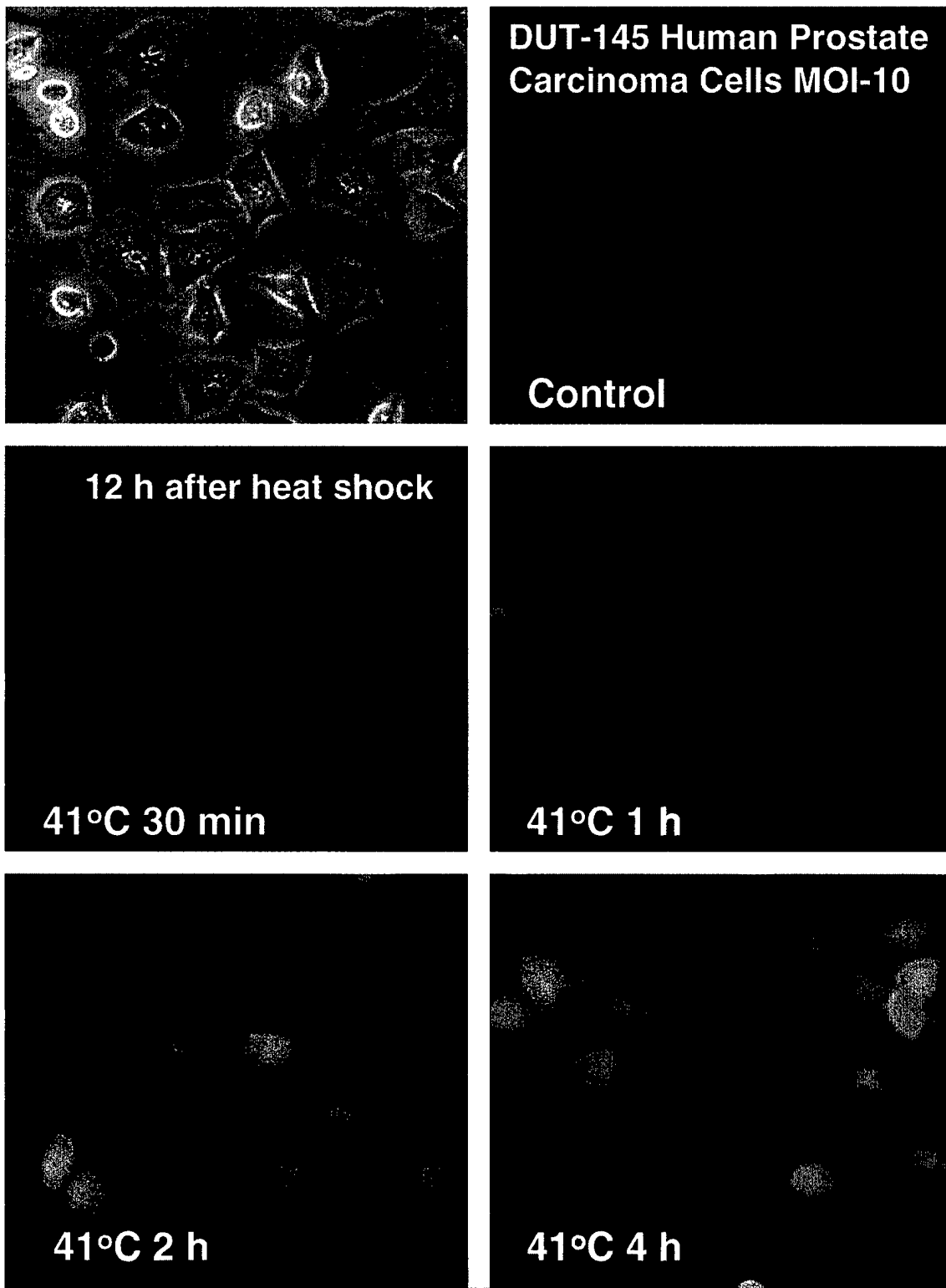


Figure 3. Experimental configuration of the rat window chamber model tumor system used to test the utility of heat shock promoter constructs for the proposed research. The heating chamber mounts directly on the microscope stage. Transmitted light, epifluorescence and phase contrast optics are available.



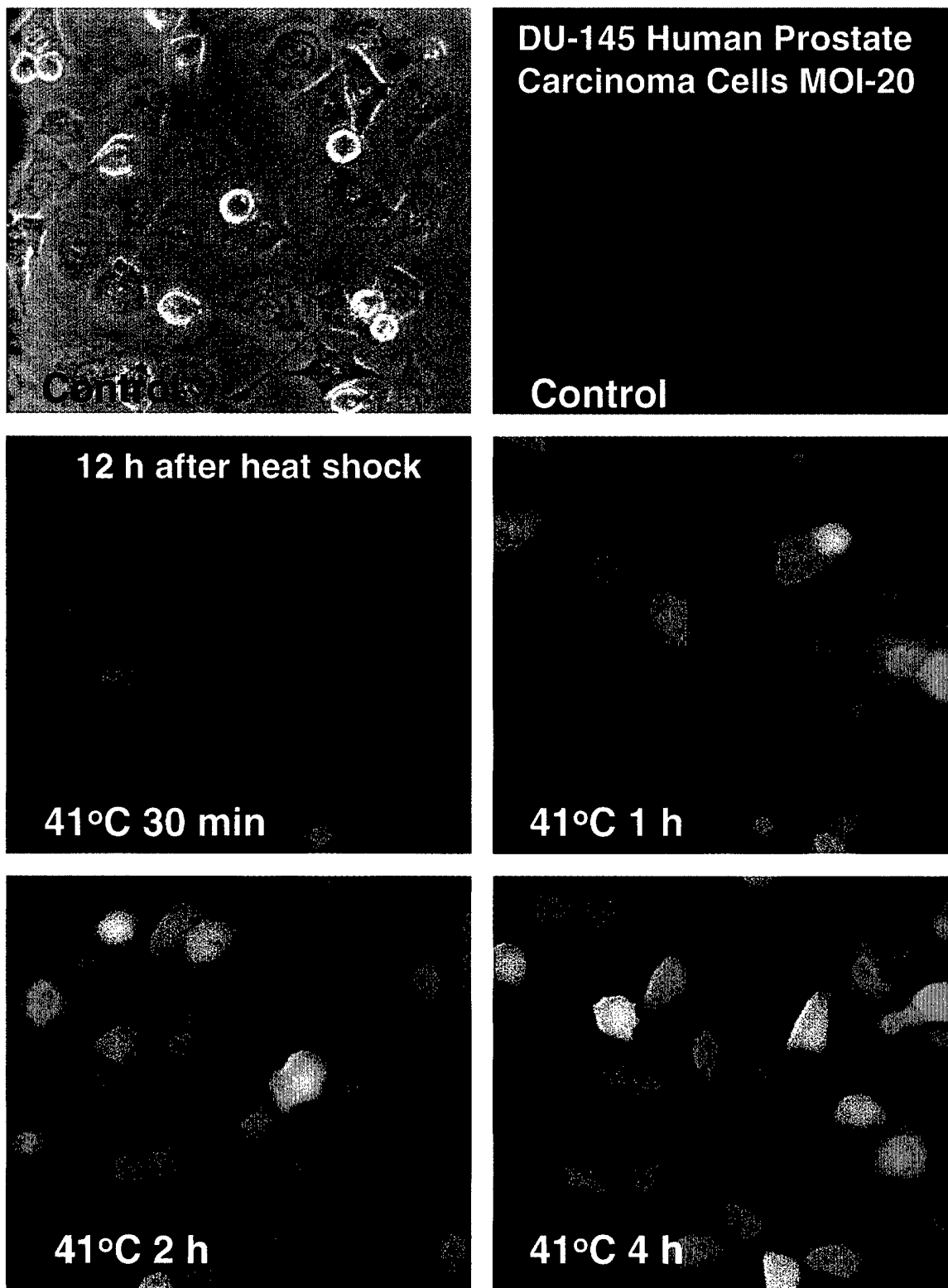
**Figure 4:** Epifluorescence photomicrographs of Rat MAC tumors grown in the window chamber and topically infected with Adv-AHSP-EGFP viral preparations. Panels A-D are for the same tumor and track EGFP expression and fluorescence as a function of time after heating for 90 minutes at 42°C. Panels E-H are for three different tumors (rats) for heat exposures of 0, 30, 60 and 90 minutes at 42°C. The tumor in H is the same one used in A-D. **A)** Immediately prior to infection **B)** 29 hours post infection and immediately prior to heating at 42°C for 90 minutes **C)** 21 hours post heating and **D)** 42 hours post heating **E)** 28 hours post infection immediately prior to heating at 42°C for 30 minutes **F)** As in E but 23 hours post heating **G)** 23 hours post heating at 42°C for 60 minutes and **H)** 21 hours post heating at 42°C for 90 minutes. The scale in the lower left portion of panel A, shows a one millimeter scale photographed through the microscope. All photographs are at the same magnification and aspect ratios are preserved. In panel D very intense fluorescence in the top portion saturated the video camera causing underexposure in the remainder of the photograph. All photomicrographs were taken at identical fluorescence excitation levels and magnification.





**FIGURE 5:**

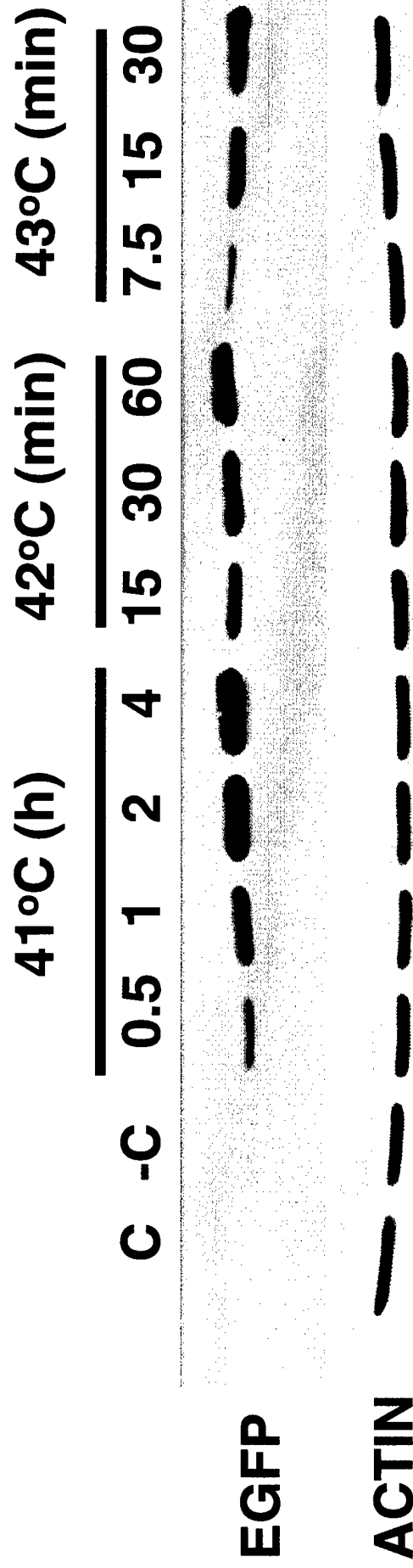
DU-145 cells were infected at MOI-10 with adenovirus vectors containing EGFP under control of the truncated heat shock promoter (virus Ad-AHSP-EGFP). Cells were heat shocked for varying times at 41.0°C 24 h following virus infection and photographed 12 h following the heat shock. Control cells were infected, sham heated and photographed at the same time as the heated cells.



**FIGURE 6:**

DU-145 cells were infected at MOI-20 with adenovirus vectors containing EGFP under control of the truncated heat shock promoter (virus Ad-AHSP-EGFP). Cells were heat shocked for varying times at 41.0°C 24 h following virus infection and photographed 12 h following the heat shock. Control cells were infected, sham heated and photographed at the same time as the heated cells.

**FIGURE 7**  
**HEAT SHOCK-INDUCED EXPRESSION OF EGFP IN**  
**DU-145 CELLS (12 h after heat shock)**  
**MOI-10 ADENOVIRUS INFECTION**



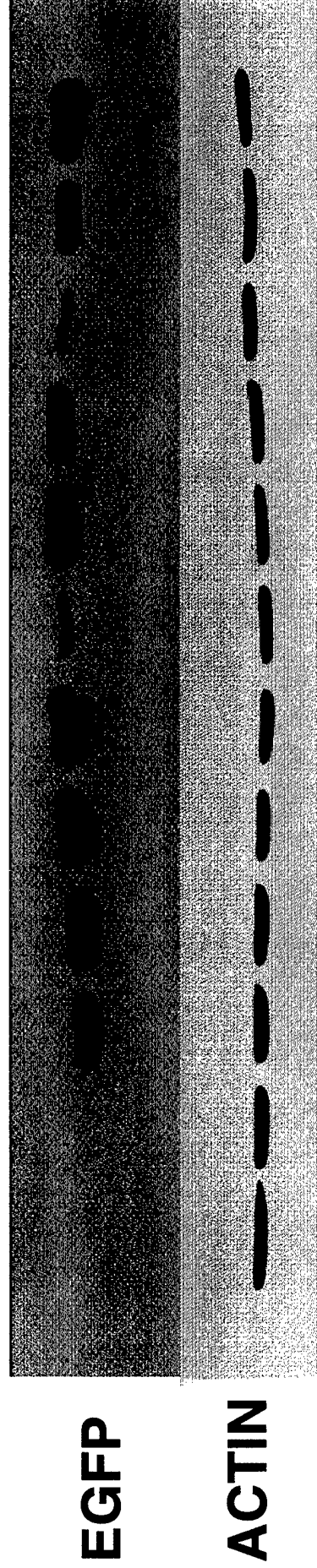
C - DU-145 cells, no virus, no heat

-C - Negative Control: Cells infected with virus having EGFP under heat shock promoter but no heat shock given



**FIGURE 8**  
**HEAT SHOCK-INDUCED EXPRESSION OF EGFP IN**  
**DUT-145 CELLS (12 h after heat shock)**  
**MOI-20 ADENOVIRUS INFECTION**

	41°C (h)					42°C (min)			43°C (min)			
	C	-C	0.5	1	2	4	15	30	60	7.5	15	30

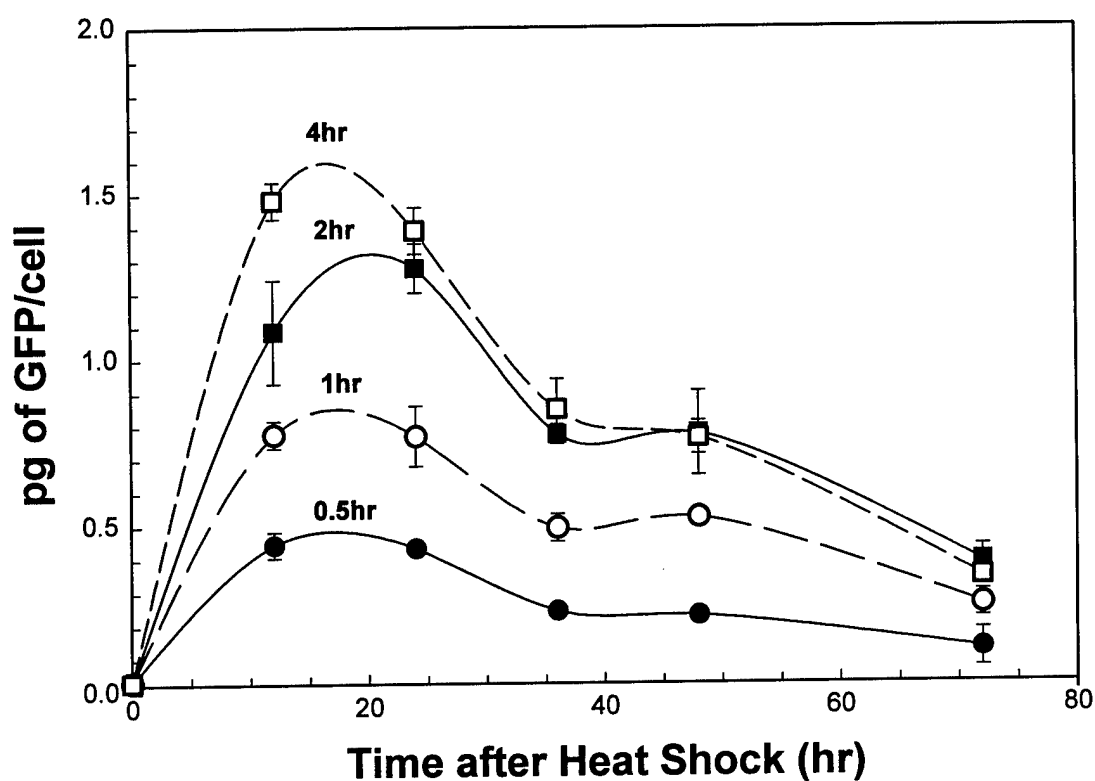


C - DU-145 cells, no virus, no heat

-C - Control: Cells infected with virus having EGFP under heat shock promoter  
but no heat shock given Negative

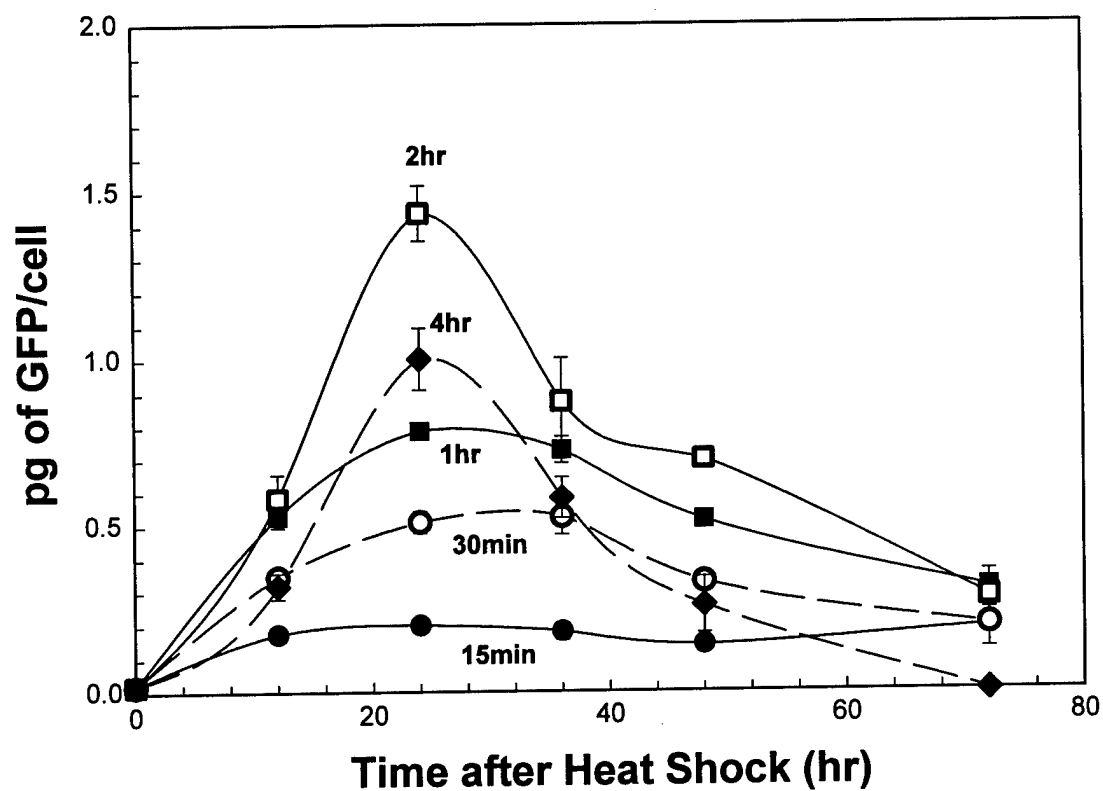
**FIGURE 9a: DU-145 Cells Infected at MOI-10 with Adenovirus Adv-AHSP-EGFP to Express EGFP following a 41.0°C Heat Shock**

pg of EGFP/cell expressed in DU-145 cells as a function of time following a 41.0°C heat shock for the indicated time. EGFP was measured by quantitative spectrofluorometry of EGFP fluorescence.

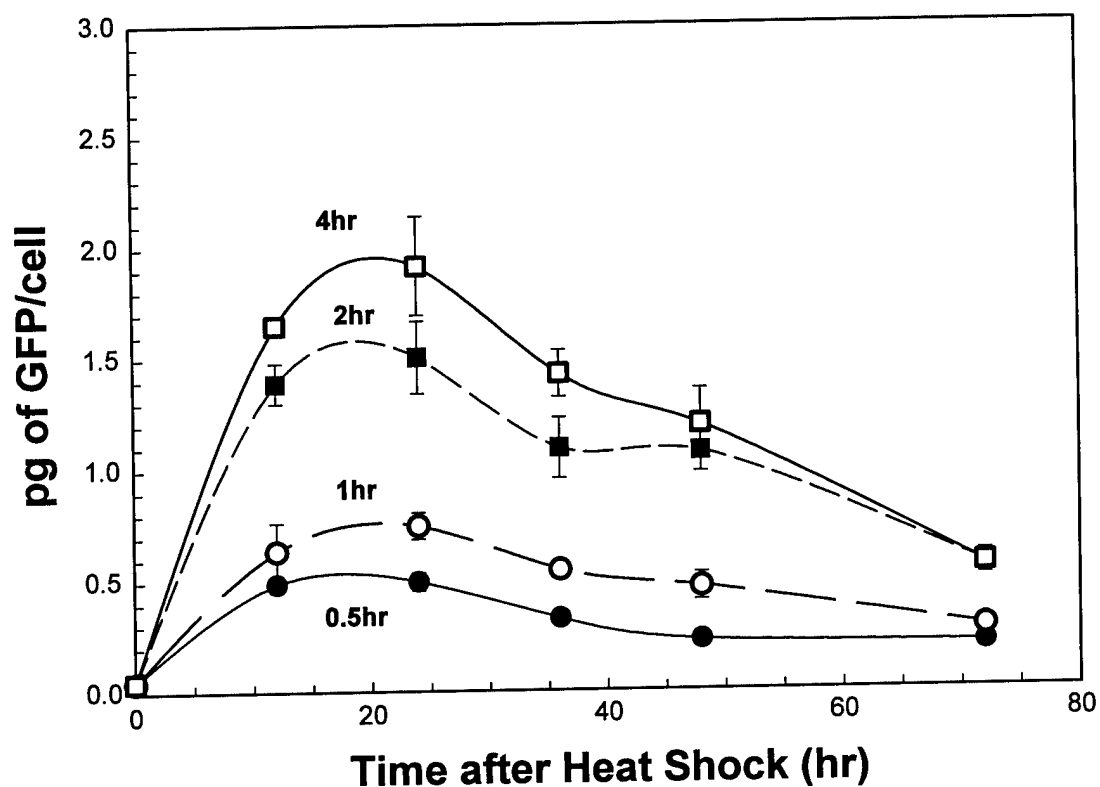


**FIGURE 9b: DU-145 Cells Infected at MOI-10 with Adenovirus Adv-AHSP-EGFP to Express EGFP following a 42.0°C Heat Shock**

pg of EGFP/cell expressed in DU-145 cells as a function of time following a 42.0°C heat shock for the indicated time. EGFP was measured by quantitative spectrofluorometry of EGFP fluorescence.

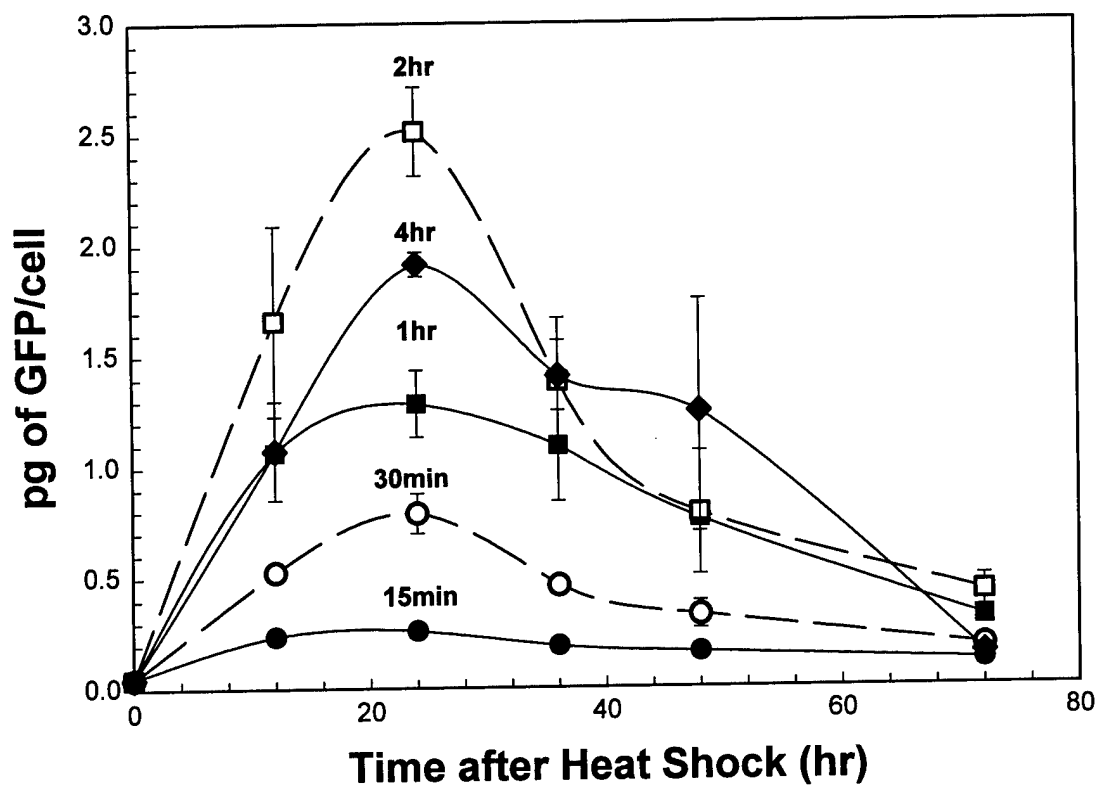


**FIGURE 9c: DU-145 Cells Infected at MOI-20 with Adenovirus Adv-AHSP-EGFP to Express EGFP following a 41.0°C Heat Shock**  
 pg of EGFP/cell expressed in DU-145 cells as a function of time following a 41.0°C heat shock for the indicated time. EGFP was measured by quantitative spectrofluorometry of EGFP fluorescence.



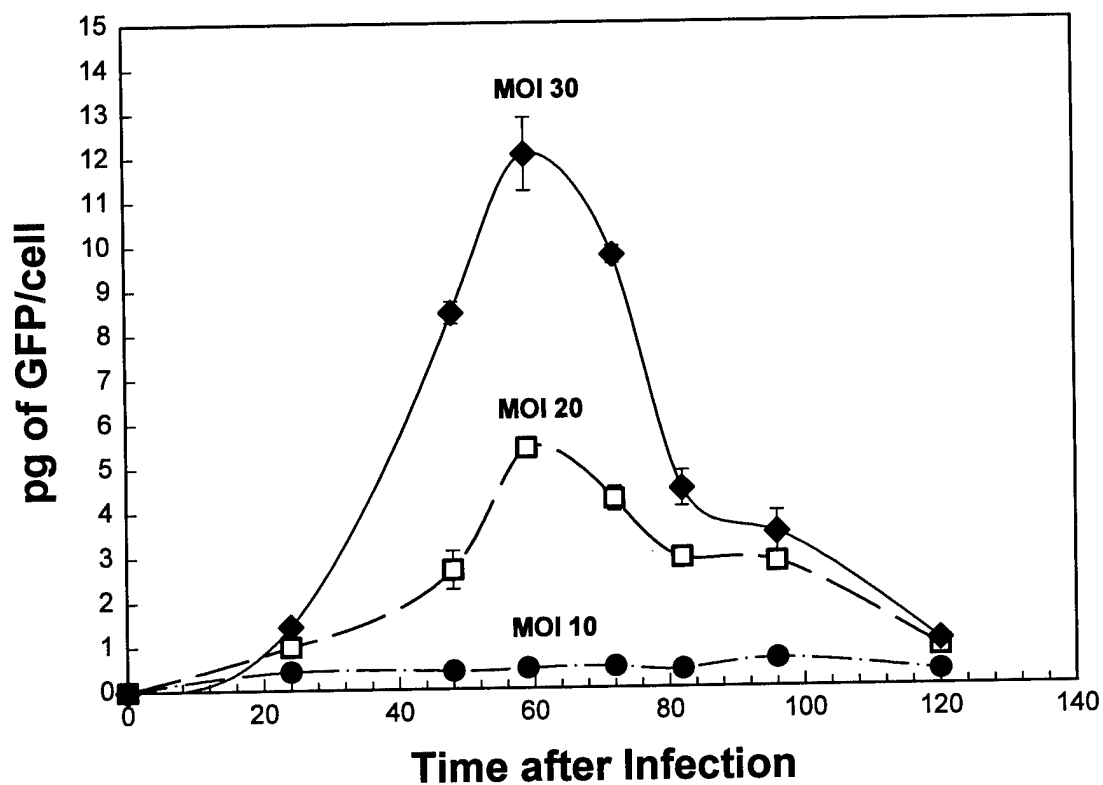
**FIGURE 9d: DU-145 Cells Infected at MOI-20 with Adenovirus Adv-AHSP-EGFP to Express EGFP following a 42.0°C Heat Shock**

pg of EGFP/cell expressed in DU-145 cells as a function of time following a 42.0°C heat shock for the indicated time. EGFP was measured by quantitative spectrofluorometry of EGFP fluorescence.



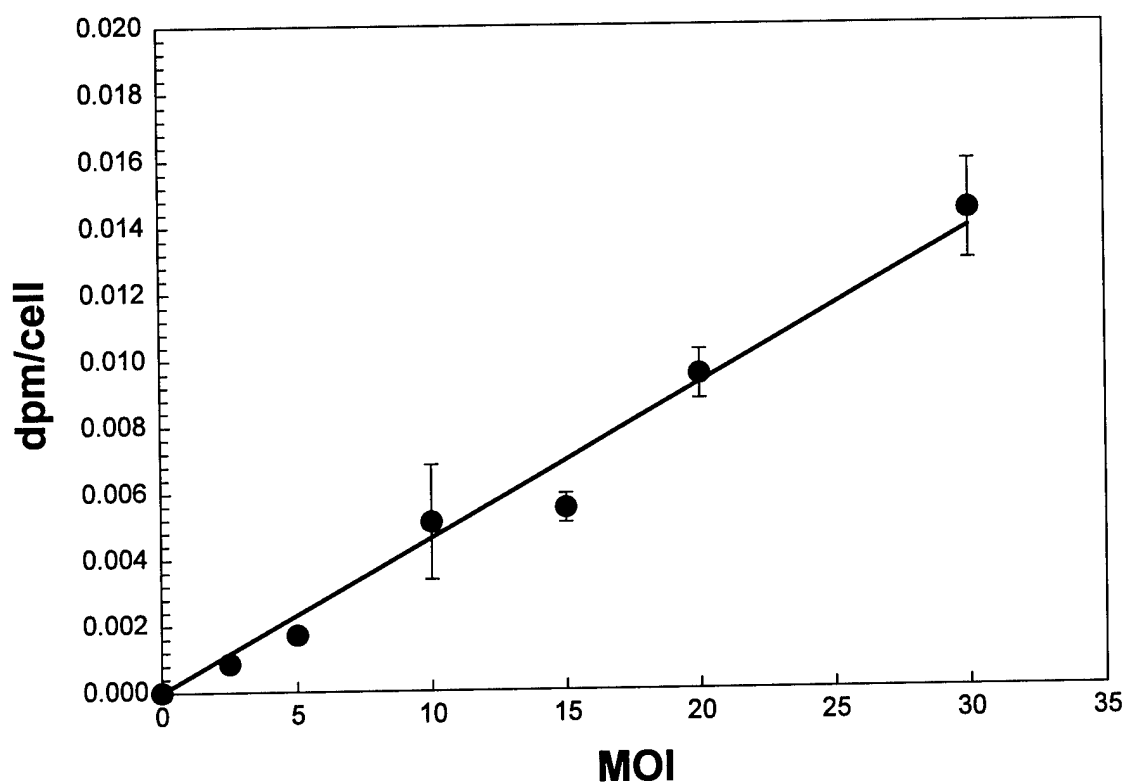
**FIGURE 10a: DU-145 Cells Infected with Adenovirus  
to Express EGFP Constitutively from the  
CMV Promoter**

pg of EGFP/cell expressed in DU-145 cells as a function of time following infection with adenovirus that introduced a construct containing EGFP expressed under control of the constitutive CMV promoter. EGFP was measured by quantitative spectrofluorometry of EGFP fluorescence.



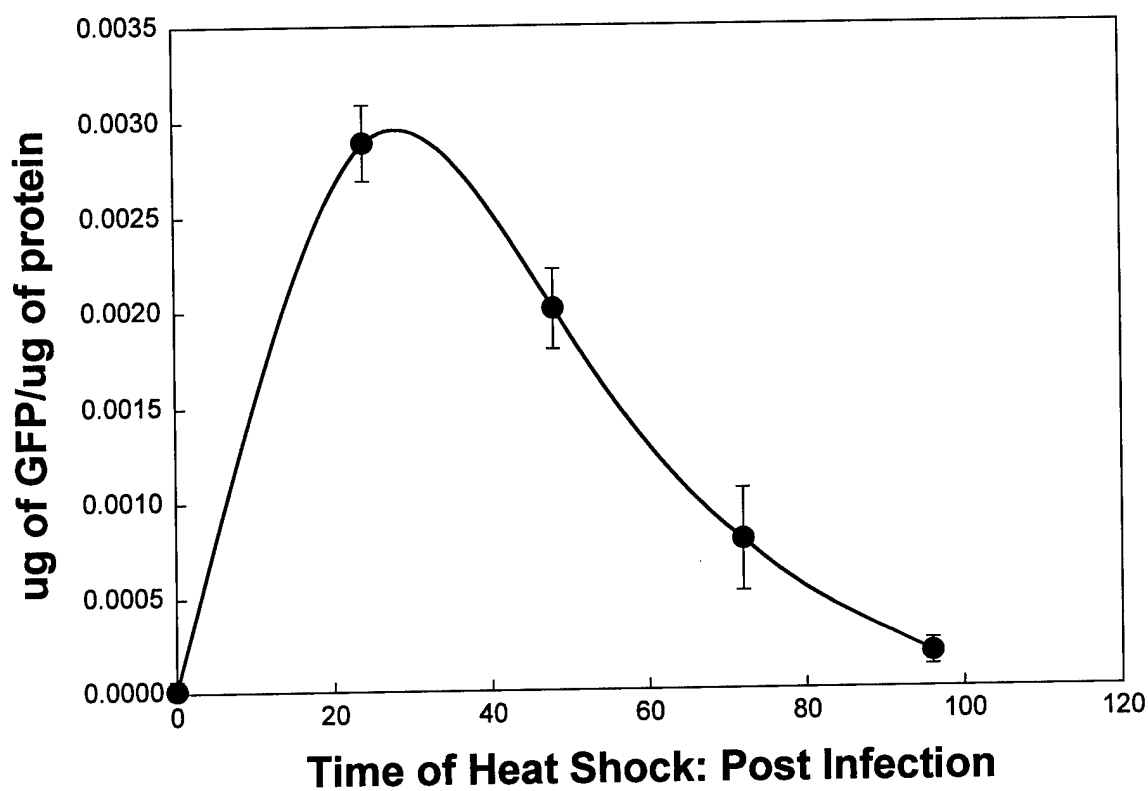
**FIGURE 10b: DU-145 Cells Infected with Adenovirus  
Labeled with  $H^3$ -Thymidine. This Virus  
Expressed EGFP from the CMV Promoter**

DU-145 cells were infected at different MOIs using an adenovirus whose DNA had been labeled with  $H^3$ -thymidine. Consequently, the measured dpm/cell was proportional to the number of virions that entered each cell. This graph clearly shows a linear relationship between the number of virions entering a cell and the MOI.



**FIGURE 11a: DU-145 Cells Infected at MOI-20 with Adenovirus Adv-AHSP-EGFP to Express EGFP following a 2 h, 41.0°C Heat Shock Delivered as a Function of Time Following Virus Infection.**

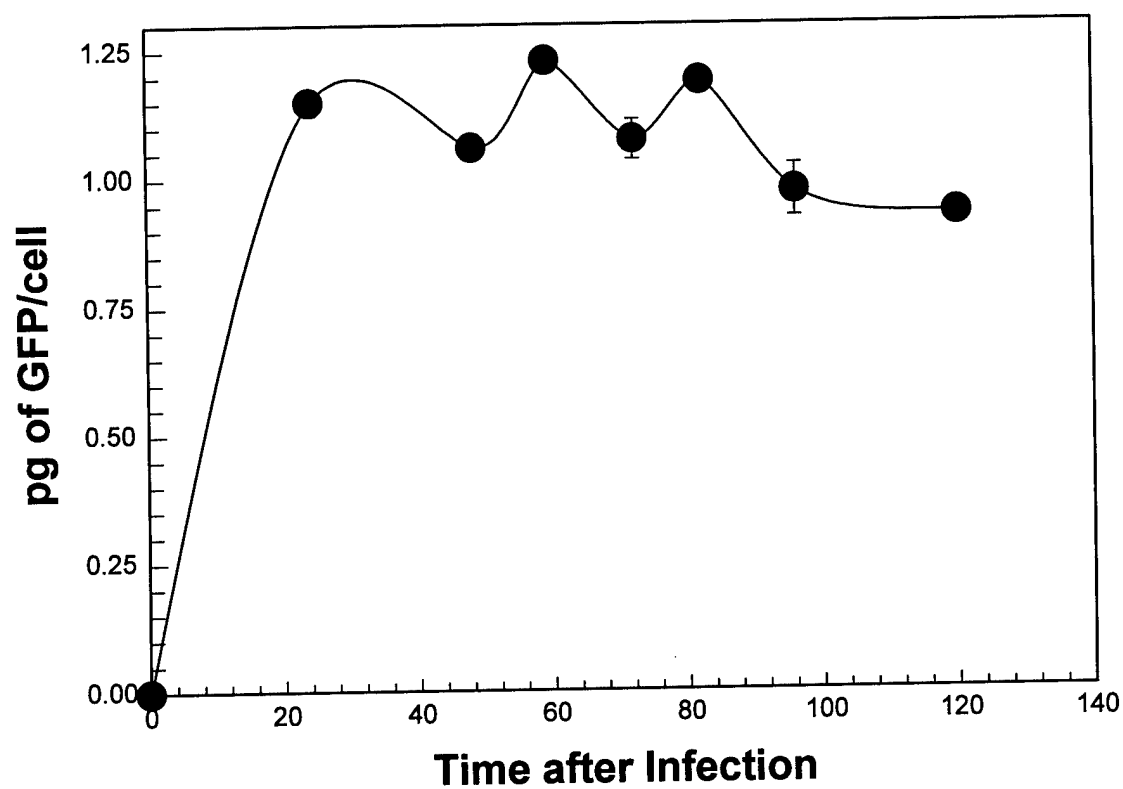
pg of EGFP/cell expressed in DU-145 cells following a 2 h, 41.0°C heat shock delivered at varying times following virus infection EGFP was measured by quantitative spectrofluorometry of EGFP fluorescence.





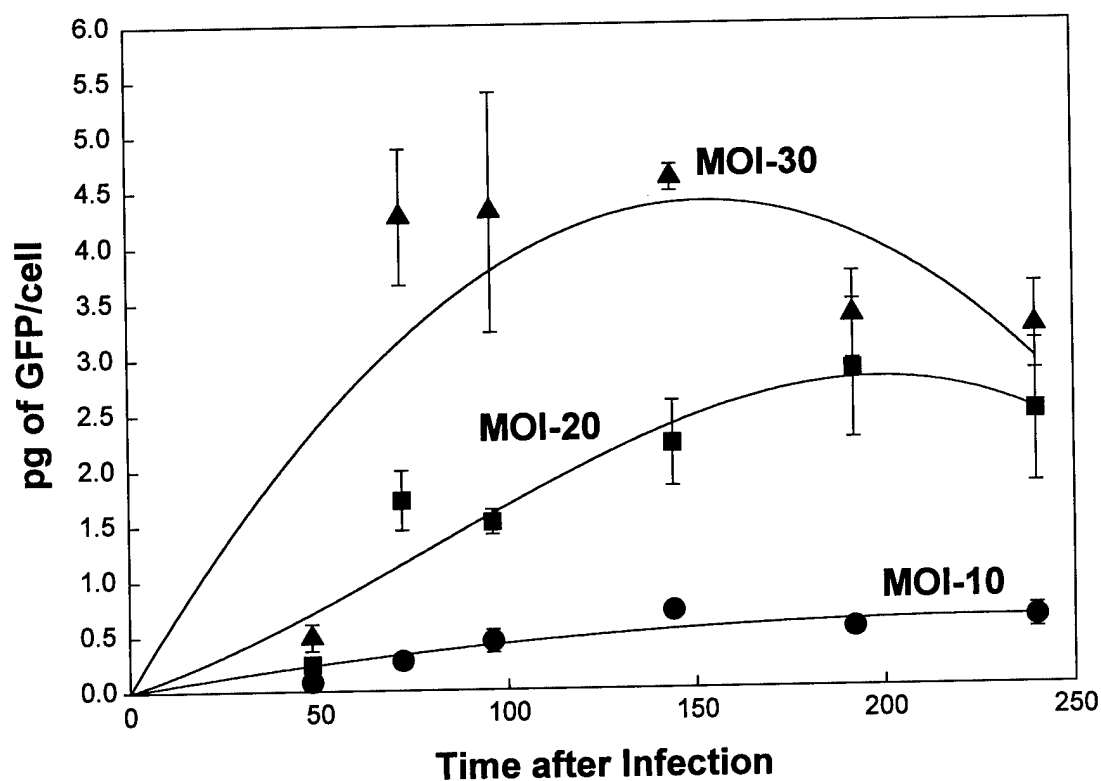
**FIGURE 11b: EGFP Expression from DU-145 Cells Heat Shocked every 12 h following Infection with Adenovirus to Express EGFP from the Heat Shock Promoter.**

DU-145 cells were heat shocked for 2 h at 41.0°C every 12 h following infection with an adenovirus that resulted in EGFP expression under control of the heat shock promoter.



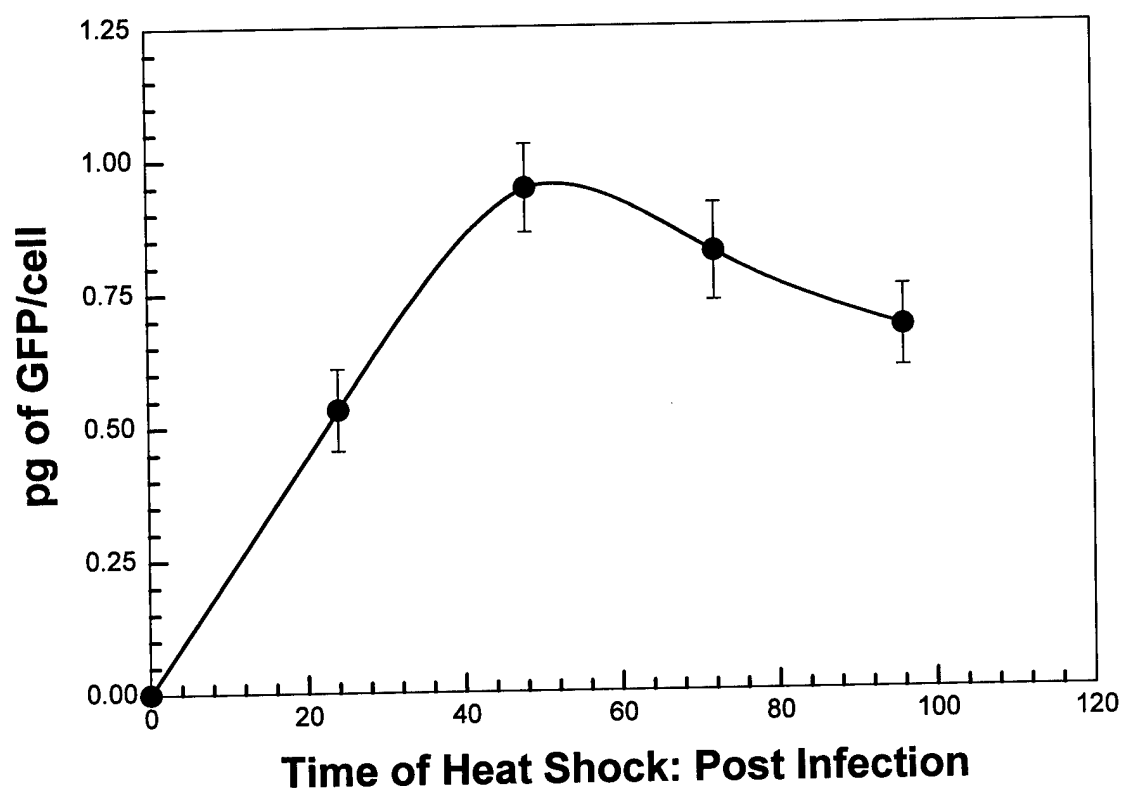
**FIGURE 12: EGFP EXPRESSION FROM THE CMV PROMOTER  
IN ADENOVIRUS-INFECTED, PLATEAU-PHASE CELLS**

Plateau phase DU-145 cells were infected at an MOI of 10, 20, or 30 and the resultant EGFP expression was quantitated as a function of time following infection. EGFP expression was lower and increased more slowly than in exponentially growing cultures. However, the duration of EGFP expression was protracted compared to that in exponential cultures.



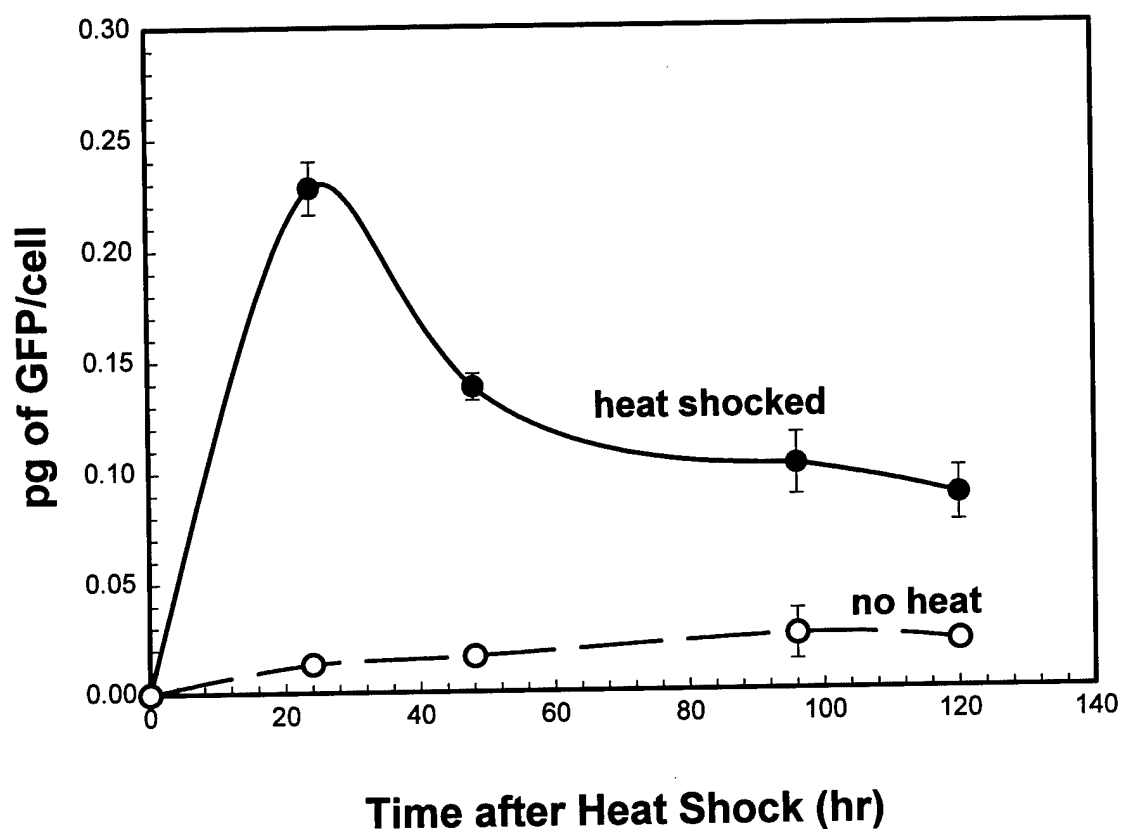
**FIGURE 13: EGFP EXPRESSION FROM THE HEAT SHOCK PROMOTER  
IN ADENOVIRUS-INFECTED, PLATEAU-PHASE CELLS. HEAT  
SHOCKS DELIVERED AS A FUNCTION OF TIME AFTER INFECTION**

Plateau phase DU-145 cells were infected at an MOI 20 and a 2 h, 41.0°C heat shock was delivered at varying times after infection to induce EGFP expression. EGFP levels were measured 24 h following heat shock, and could be induced at later post-infection times than in exponentially growing cells.

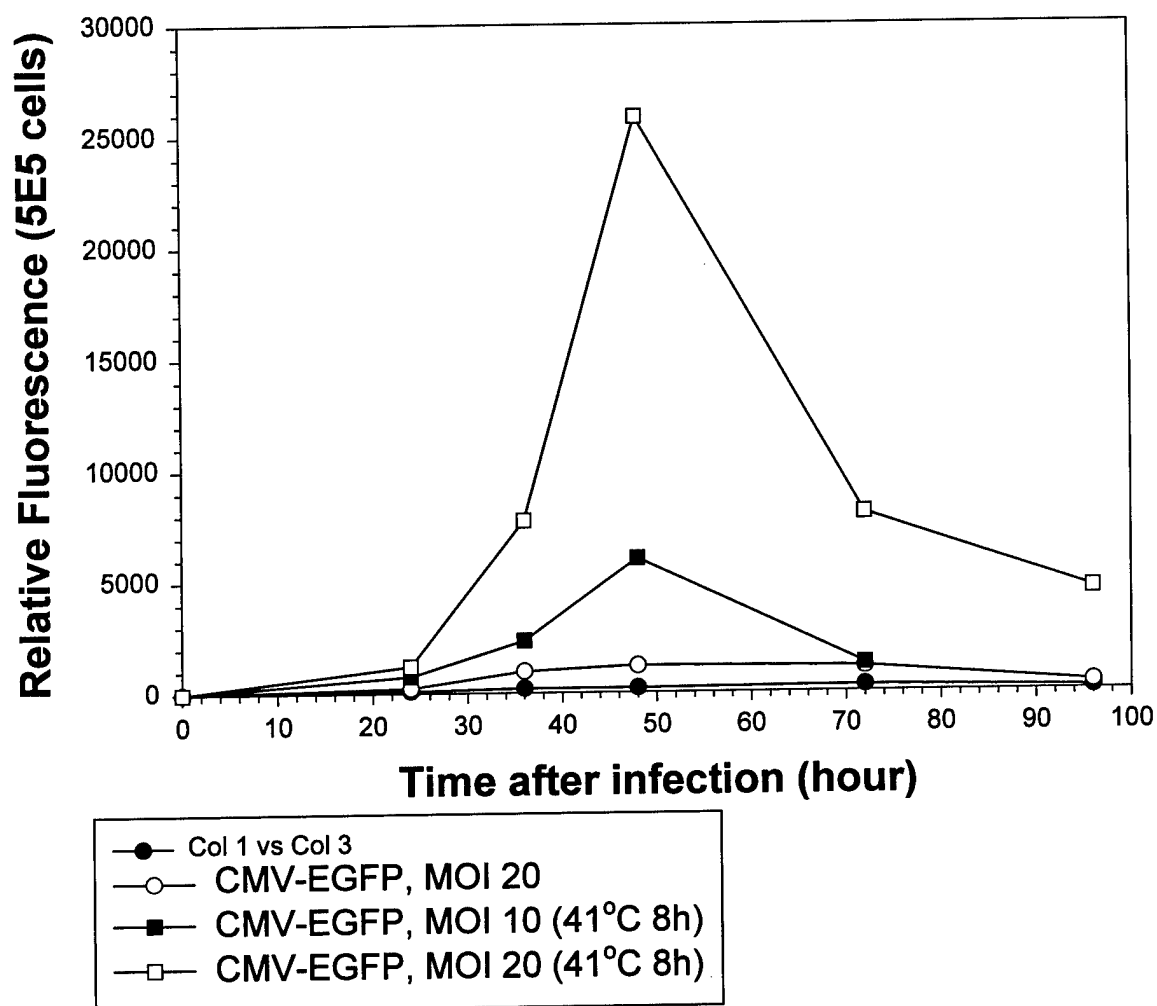


**FIGURE 14: EGFP Expression from the Heat Shock Promoter in Adenovirus-Infected, Plateau Phase Cells . EGFP was Measured as a Function Time after a Heat Shock Delivered 24 h Post-Infection.**

Plateau phase DU-145 cells were infected at an MOI 20. A 2 h, 41.0°C heat shock was delivered at 24 h post-infection, and EGFP levels were measured at varying times later. Interestingly, the decay of EGFP from its peak level was similar to that in exponentially growing cells.

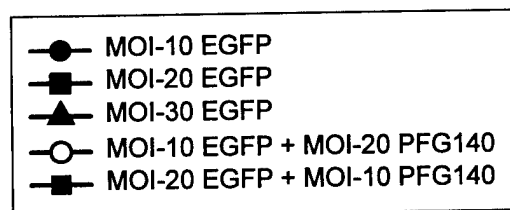
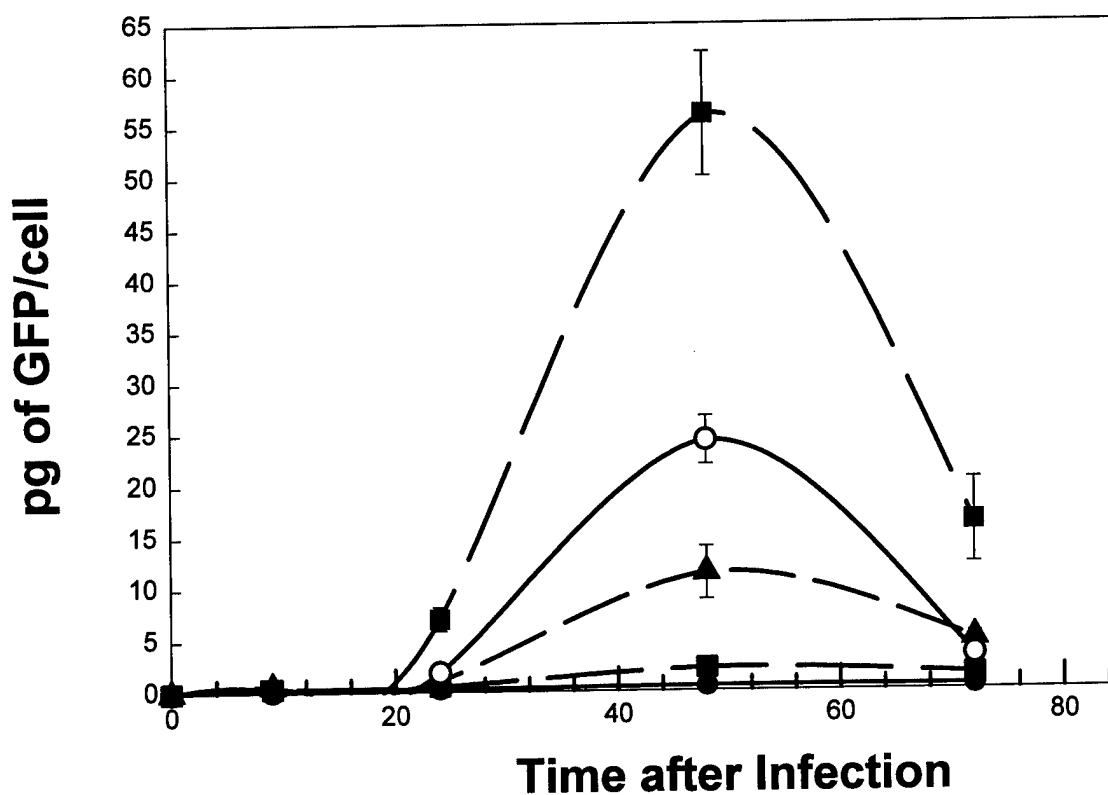


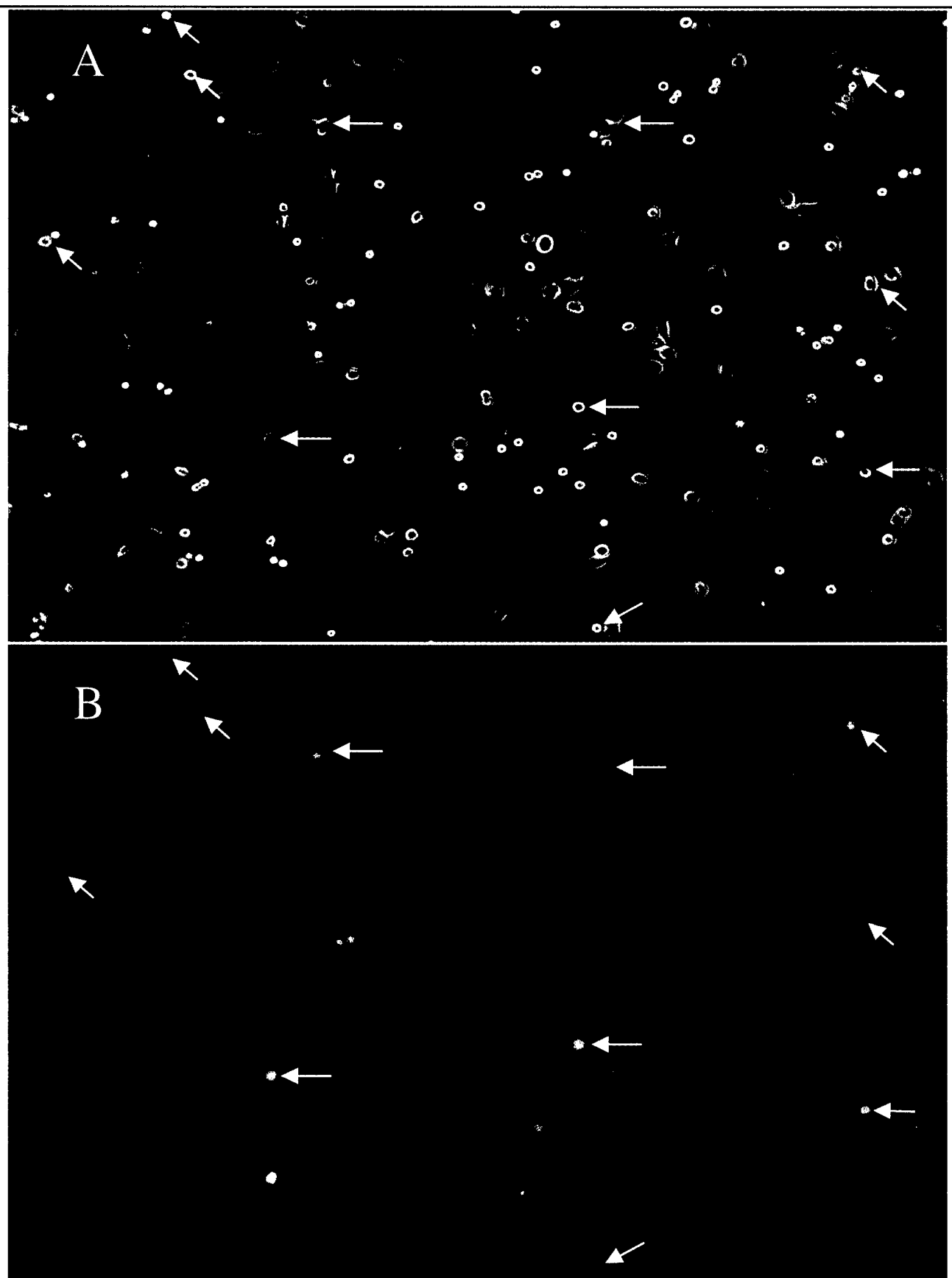
**Figure 15: Heat Shock Potentiation of CMV Promoter Activity**  
 DU-145 cells were infected at an MOI of 10 or 20 with adenovirus to express EGFP under control of the CMV promoter. Ten hours after infection, some infected cells were heat shocked at 41.0°C for 8 h and then returned to 37.0°C. Expressed EGFP was measured periodically thereafter. The heat shock markedly increased CMV promoter activity.



**FIGURE 16: PFG-140 Virus Enhancement of Transgene Expression  
by the CMV Promoter.**

DU-145 cells were infected at different MOIs with adenoviruses to express EGFP by the CMV-promoter. Adding in PFG-140 (total MOI of 30) markedly increased EGFP Expression.





**FIGURE 17.** A549 lung cancer cells transfected (electroporated) with a plasmid containing the DNA for a fusion protein with the EGFP and DTXA genes (CMV-EGFP-DTXA). A) Phase contrast image and B) the epifluorescence image taken eight hours after the cells were transfected. Approximately 65% of the cells fluoresced to some extent and all fluorescing cells died within 36 hours. Most cells not fluorescing proliferated normally (see discussion in text).

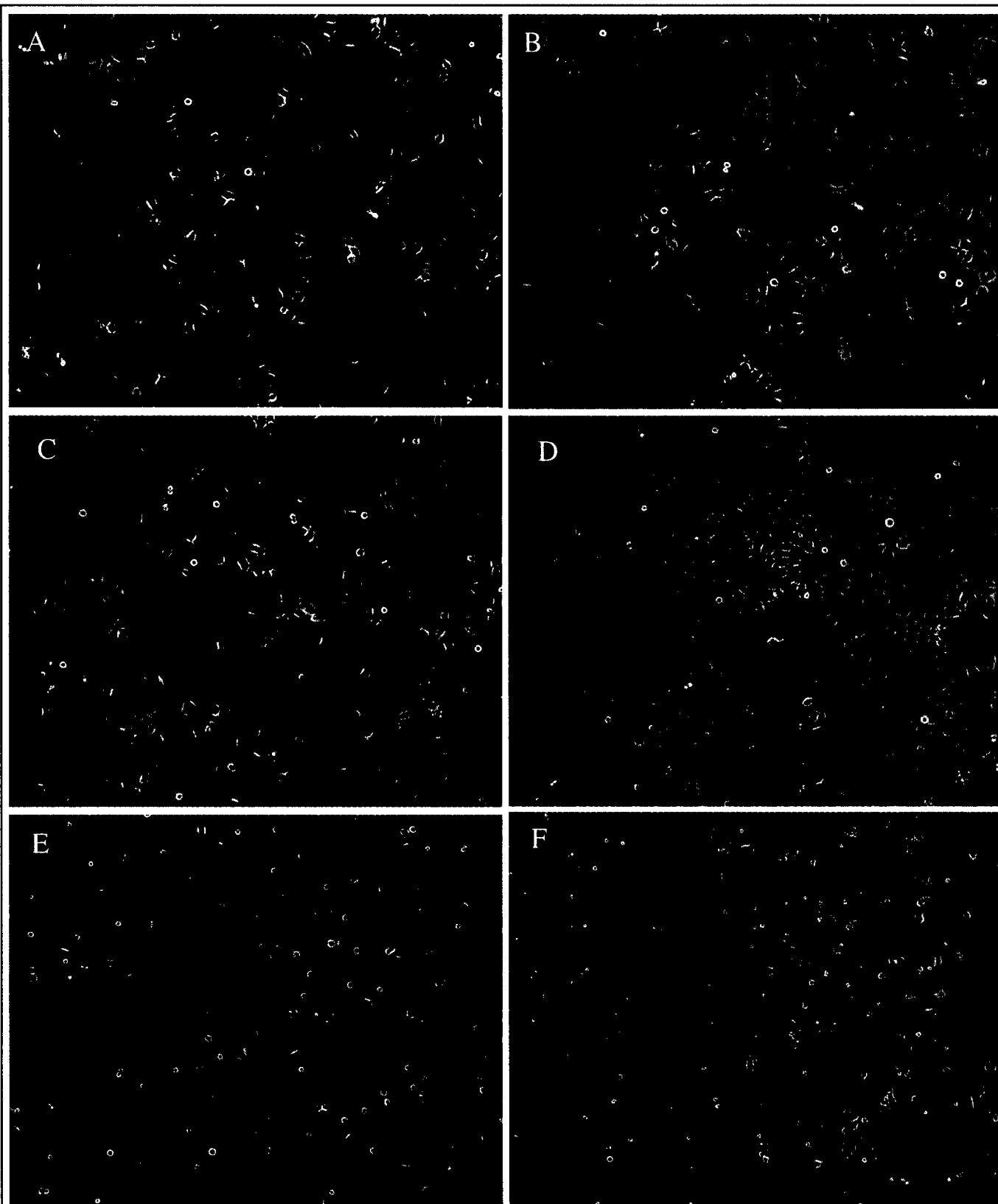


Figure 18: A549 lung cancer cells transfected with two different plasmids. A) Three hours after transfection with a CMV-EGFP construct, B) Identical field in A 36 hours after heating for 3 h at 43.0°C. C) Transfected with the AHSP-DTXA plasmid and imaged 3 h after transfection (no heat) D) 36 hours later (no heat). E) Transfected as in C but imaged 1 h following 3 h at 41.0°C. F) Identical field as E but 36 hours after heating. A-C show normal proliferation. Panel E shows significant cell kill by 1 h after heating with continual cell loss through 36 hours (Panel F).